STRUCTURAL AND IMMUNOLOGICAL DOMAIN ANALYSIS OF THE CARBOXYTERMINAL TAILS OF THE HIGH MOLECULAR WEIGHT NEUROFILAMENT SUBUNIT PROTEINS NF-M AND NF-H

By

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By

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Unlike the smooth 10 nm intermediate filaments (IFs) found in the cytoskeleton of most cell types, neurofilaments (NFs), the IFs of neurons, have side-arms projecting out radially from a 10 nm core filament. The projection arms are thought to contain the long carboxyterminal tails which are unique to the middle (NF-M) and high (NF-H) molecular weight neurofilament subunit proteins. The tails of NF-M and NF-H appear to contain several domains based upon amino acid sequence analysis. This study reports on the construction of cDNA subclones containing these putative domains from rat NF-M and NF-H, the expression and purification of the corresponding fusion-proteins, the production and characterization of polyclonal and monoclonal antibodies against these putative domains, as well as the histological and pathological distribution of NF-M and NF-H using these antibodies.

Epitopes for a large number of widely used monoclonal neurofilament antibodies were mapped to these putative domains including the commercially available antibodies NN18 and N52. Several unusual findings were made concerning the immunohistochemical distribution of NF-M and NF-H in the rat nervous system. We describe a novel cell type in the granular layer of the vestibulocerebellum which is strongly immunoreactive for NF-H, but not for NF-M. We demonstrate NF reactivity in a subpopulation of cerebellar parallel fibers, previously thought not to contain NFs. The type I spiral ganglion neurons are shown to be strongly immunoreactive for NF-M, but not for NF-H. An immunological masking event on a specified segment of NF-M is demonstrated to begin at postnatal day 4 in rat myenteric neurons. In addition, an immunohistochemical examination of pathological human brains revealed that the carboxyterminal tails of NF-M and NF-H are present in the Lewy body inclusions which are pathognomonic for Parkinson's disease, but are unlikely to be specific components of the neurofibrillary tangles found in Alzheimer's disease.

CHAPTER 1
INTRODUCTION AND BACKGROUND

Cytoskeletal Networks

Three major cytoskeletal networks are known to exist in most mammalian cells: the microfilaments composed of polymerized actin, the microtubules consisting of polymerized tubulin and the intermediate filaments (IFs), which are assembled from various cell-type specific subunits. The roles of the microfilamentous and microtubular networks have been widely characterized with respect to many cellular processes including growth, motility, mitosis, intracellular trafficking and production of tensile strength. The function of intermediate filaments, however, still remains elusive. For lack of other evidence, these structures are often assigned a mechanical role. Because different subunit proteins are utilized in different cell types, it has been hypothesized that intermediate filaments are involved in determination or maintenance of cell shape. Because these cell-type specific subunits are biochemically and immunologically distinct, they have proven to be useful cell-type specific markers during development and oncogenesis when morphological characteristics are often indiscriminate. Intermediate filament subunits include: the various acidic and basic keratins found in

epithelia, desmin in muscle, glial fibrillary acidic protein (GFAP) in astrocytes and Bergmann glia and vimentin in mesenchymal cells, as well as an increasing number of neurofilament protein subunits; it is of note that the lamins, which comprise a major part of the scaffolding found inside the inner nuclear membrane of all eukaryotic cells, are also considered members of the intermediate filament family based on protein sequence (McKeon et al., 1986). A classification scheme to group different intermediate filament subunit proteins has been proposed which is based upon sequence similarity and intron placement (Steinert and Roop, 1988); Table 1-1 is an updated modification of this classification scheme.

Mammalian Neurofilament Subunit Proteins

The 10 nm diameter neurofilaments (NFs) seen in the electron microscope are now known to correspond to the neurofibrils seen in the light microscope following the use of silver stains. Using ultrathin section electron microscopy neurofilaments can be distinguished from the intermediate filaments found in other cell types, including glia, due to the presence of periodic fine projections protruding between filaments within bundles; these protrusions can be used to differentiate between processes of neurons and glia.

Technically a genuine neurofilament subunit protein is distinguished from a neurofilament associated protein based

Table 1-1. MAMMALIAN INTERMEDIATE FILAMENT PROTEIN CLASSIFICATION BASED UPON CONSERVATION OF INTRON SPLICE SITES

Class	Name
I	acidic keratins
II	basic keratins
III	vimentin desmin glial fibrillary acidic protein (GFAP) peripherin
IV	NF-L NF-M NF-H α-internexin
V	nuclear lamins
VI	nestin

on its ability to actually incorporate into the neurofilament backbone in vivo. Until recently the neurofilament protein subunits were referred to as a "triplet" because it was thought that only three subunits existed, namely NF-L, NF-M and NF-H; these subunits are so named because of their relatively low, middle or high molecular weight. The triplet proteins were originally described as major components of slow axonal transport with apparent molecular weights by SDS-PAGE of approximately 68, 145 and 200 kDa (Hoffman and Lasek, 1975; Liem et al., 1978). With complete sequence information it now appears that the actual molecular weights should be 60, ~95 and ~115 kDa; this discrepancy in calculated and observed molecular weight is apparently due to secondary structure influenced to a large degree by multiple phosphorylation sites

in the large carboxyterminal tails (Julien and Mushynski, 1982; Kaufmann et al., 1984).

Although the triplet proteins are still thought to be the predominant neurofilament subunits of the adult (i.e., post-mitotic) nervous system (Tapscott et al., 1981), other neurofilament subunit proteins seem to be more prevalent at earlier developmental stages. Vimentin is found developmentally in mesenchymal cells and dividing neuroepithelial cells and is often coexpressed with neurofilament triplet subunits during post-mitotic development. In the adult vimentin is seen in most cells of mesenchymal origin as well as in reactive microglia and even in some mature neurons (Dräger et al., 1984; Shaw and Weber, 1983; Schwob et al., 1986); therefore, vimentin could be classified as a genuine neurofilament subunit. Two more recently described neurofilament subunits named α-internexin (66kD) (Pachter and Liem, 1985) and peripherin (57kD) (Portier et al., 1984; Parysek and Goldman, 1987; Leonard et al., 1988) have somewhat complementary distributions in the developing central (CNS) and peripheral (PNS) nervous systems, respectively. Expression of α-internexin appears to precede that of the triplet subunits in the CNS and can also be found in neuronal cell types previously thought not to possess neurofilaments (Kaplan et al., 1990). In addition, peripherin has now been found to have two additional, though less abundant, forms derived from differential mRNA splicing

(Landon, et al., 1989). Another newly described neural intermediate filament protein, nestin, is found in neuroepithelial stem cells (radial glia and their developing progeny) and appears to be quite distinct in sequence, structure, intron pattern and size from other neurofilament protein subunits (Lendahl et al., 1990).

Mammalian NF Protein Structural Features

Structural characteristics of all intermediate filaments (IFs) include a globular amino-terminal head, a central α-helical rod and a carboxyterminal tail. The central rod region is conserved in all IF subunits. Filament assembly is thought to involve the rod region, which always contains long heptad repeats of large hydrophobic amino acids with relatively rare interruptions. This conserved heptad repeat structure allows the α-helical regions of two subunits to be stabilized by intercalation of hydrophobic residues forming an α-helical coiled-coil (Crick, 1953). Alpha-helical coiled-coils are also found in dimerized molecules like myosin heavy chains, tropomyosin and the DNA binding proteins c-*fos* and c-*jun*. The two major heptad breaks, called linkers or spacers, are usually created by proline residues and define the borders between coil 1a, coil 1b and coil 2 (see Figure 1-1). Nestin, NF-M and NF-H do not appear to contain the first linker such that they may have an unbroken coil 1 (Lees et al., 1988; Myers et al., 1987). Two heptad "stutters" are

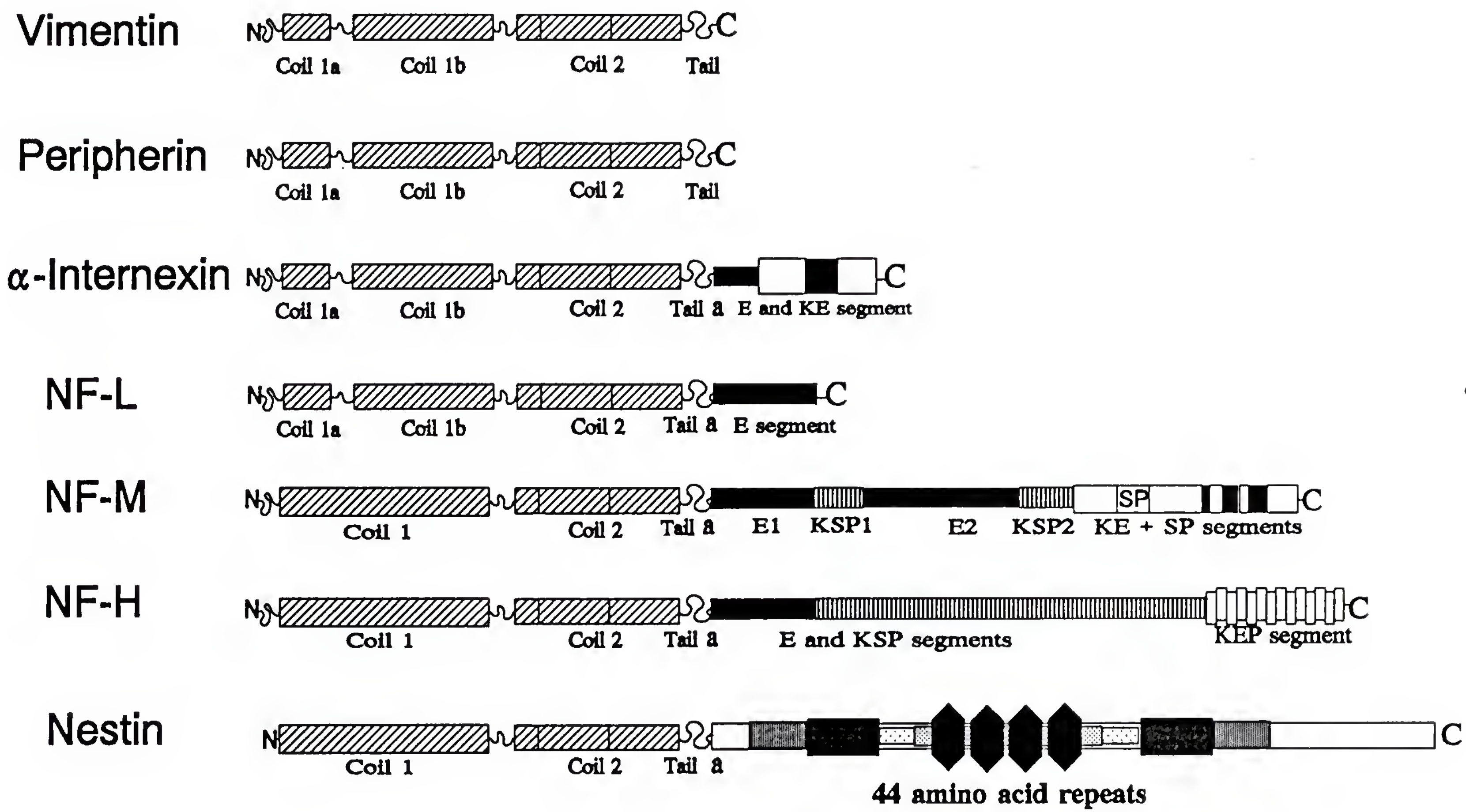

Figure 1-1. Diagram of the domain structures of NF subunit proteins

consistently found near the beginning of and about three-fifths through coil 2 due to a single residue insertion, thus shifting the heptad repeat out of register. Further comparison of the rod domains across IF protein subunits reveals that the α-helical regions are almost invariant in length, whereas the linkers are of somewhat variable length. The amino-terminal region of coil 1a and the carboxyterminal end of coil 2 are very highly conserved in amino acid sequence. This high conservation of coil 2 region accounts for the ability of an antibody (α-IFA) described by Pruss et al. (1981) to detect all known IF protein subunits (Geisler et al., 1983).

Across all IFs the head and tail regions are most variable, though strong similarities in these segments can be found in members within IF classes and occasionally between IF classes (see Table 1-1 and Figure 1-1). The globular heads predominantly contain β-sheets and β-turns which have prolines and small neutral amino acids; in class III and IV IF proteins the head also has an abundance of arginine (Geisler and Weber, 1982). The most dramatic feature of the high molecular weight class IV neurofilament proteins, however, is their large and apparently multi-domain carboxytails (Geisler et al., 1984, 1985a and 1985c). The rod domains of NF-L, NF-M and NF-H are assumed to form the central filament core. The NF-M and NF-H tail extensions are thought to account for the thin fibrous protrusions visualized by ultrathin section electron

microscopy (Willard and Simon, 1981; Sharp et al., 1982; Hirokawa et al., 1984; Hisanaga and Hirokawa, 1988; Mulligan et al., 1991). These carboxy-tail regions are proposed to contain the domains in which neurofilament-specific functions would reside; one such domain which contains repetitive sequences rich in lysine, serine and proline (KSP) is known to be the predominant site of phosphorylation in these molecules (Shaw, 1991 for review).

Neurofilament Assembly

Although the details of intermediate filament assembly are still controversial, the consensus is that α-helical coiled-coil dimers associate laterally to form tetramers and octomers. Questions of polarity and alignment, as well as which oligomeric unit or units might serve as a protofilament structure capable of incorporation into a filament, are, however, still topics of debate (Traub et al., 1992; Hisanaga and Hirokawa, 1990; Hisanaga et al., 1990b; Tokutake, 1990b; Steinert and Roop, 1988; Geisler et al., 1985b). Attempts to dissect and recreate the assembly process of neurofilaments (and other intermediate filaments) have made use of in vitro reconstitution techniques. Subunit proteins are typically purified by DE-52 column chromatography in the presence of 6M urea (Tokutake, 1984) and then dialyzed to remove the denaturants. When renaturation is complete most 10nm filament subunits self-polymerize to form filaments. From such

experiments it seems that pure NF-L, but not NF-M or NF-H alone, is capable of homopolymerization into normal appearing filaments; NF-M and NF-H can, however, co-assemble with NF-L (Geisler and Weber, 1981; Liem and Hutchison, 1982; Zackroff et al., 1982) and have been reported to homopolymerize into shorter than normal, rough-surfaced filamentous structures (Tokutake et al., 1984; Tokutake, 1990). Experiments attempting to study assembly in cultured cells have utilized DNA transfection techniques to place normal or mutagenized subunits that are not usually found in the cell type being used (Chin et al., 1991; Chin and Liem, 1989) These studies also find that NF-M and NF-H are not capable of homopolymerization, but can use a native type III IF network for heteropolymerization. It has been suggested that the long carboxytails, which contain multiple phosphorylation sites, interfere with the homopolymerization of NF-M and NF-H (Liem and Hutchison, 1982; Wong et al., 1984; Wong et al., 1990); this premise implies that NF-M and NF-H can readily form heteropolymers with NF-L because NF-L effectively dilutes out the ratio of tail to rod domains. Alternatively, it has been suggested that heteropolymerization could be an inherent property of NF-M and NF-H, analogous to the pattern seen with the pairing of acidic and basic keratins (Shaw, 1991). The arginine-rich head regions appear to be important for intermediate filament assembly (Nelson and Traub, 1983; Kaufman et al., 1985; Geisler and Weber, 1988) and have been

shown to interfere with filament assembly if they are mutagenized in transgenic mice (Gill et al., 1990; Wong and Cleveland, 1990). Indeed phosphorylation at key sites bordering the conserved rod domain is likely to be the endogenous method for controlling disassembly of filaments within a cell (Hisanaga et al., 1990a; Ignaki et al., 1987). Peripherin and α-internexin are each apparently capable of homopolymerization into 10 nm filaments (Parysek and Goldman, 1987; Chiu et al., 1989).

Neurofilament Involvement in Disease

Although no direct causal role has been firmly established with a naturally occurring neurofilament defect, various aberrations in neurofilament morphology, phosphorylation-state and distribution have been associated with a variety of neuropathologies. The most obvious correlation between neurofilaments and neuropathology is the phosphorylation of perikaryal neurofilaments in response to axotomy (Dräger and Hofbauer, 1984) or other forms of neurodegeneration (Sternberger et al., 1985). When this phenomenon reverts in systems that can fully regenerate, such as that following the initial stages of regeneration, the perikaryal neurofilaments are no longer phosphorylated (Moss and Lewkowicz, 1983; Shaw et al., 1988). Neurofilamentous accumulations have been associated with intraneuronal inclusions found in Alzheimer's disease, progressive

supranuclear palsy (PSP), Pick's disease, Parkinson's disease, Guamanian amyotrophic lateral sclerosis (ALS) and giant axonal neuropathy (GAN) (Gambetti et al., 1983; Goldman et al., 1983; Matsumoto et al., 1990; Asbury et al., 1972; for review see Goldman and Yen, 1986). Although it is still debatable, early immunological data suggested that neurofilament epitopes were present in the paired helical filaments (PHF), a major component of the neurofibrillary tangles (NFTs) from Alzheimer's disease. Many studies now indicate that this signal may have been largely due to cross-reactivity of the NF KSP region with a similar epitope of the microtubule associated protein (MAP), tau (Miller et al., 1986; Ksiezak-Reding et al., 1987; Schmidt et al., 1990), which has been shown by biochemical methods to be a PHF component (Goedert et al., 1988).

Putative Functions

Despite the fact that neurofilaments are one of the most prominent features of the neuronal protein profile (at least 10% of total protein in the central nervous system), their function is still elusive. Arguments have been made for mechanical roles in providing strength and support, as well as controlling axon diameter (Hoffman et al., 1984, 1987). Phosphorylation effects on the carboxytails have been implicated as a potential regulatory mechanism to alter the spacing between filaments via the sidearm projections

(Hisanaga and Hirokawa, 1989). A recently described mutant strain of Japanese quail (quiverer) which apparently lacks neurofilaments shows a significantly reduced axon diameter (Yamasaki et al., 1991). Transgenic mice which overexpress NF-L, however, do not produce axons of increased caliber (Monteiro et al., 1990). The clarification of this relationship between neurofilaments and axonal structure will require further investigations of both the mutant and transgenic animal models.

Unlike the microfilamentous and microtubular networks which have many dedicated associated proteins (such as their respective myosin and kinesin motor protein families), neurofilaments lack well-documented associated proteins. Considering that most microfilamentous and microtubular functions rely heavily upon interactions with their associated proteins, it is also likely that our understanding of neurofilament function will not grow considerably until we isolate and study neurofilament associated proteins. Despite the absence of obligate neurofilament associated proteins, some interactions have been reported. Neurofilaments have been shown to bind to microtubules via the microtubule-associated proteins MAP2 and tau (Runge et al., 1981; Leterrier et al., 1982; Minami et al., 1982; Minamai and Sakai, 1983; Heimann et al., 1985; Miyata et al., 1986), as well as to microfilaments via the actin-associated protein fodrin/spectrin (Frappier et al., 1987). Steiner et al.

(1987) have shown that NF-L interacts with the synaptic vesicle phosphoprotein synapsin 1. Although it has yet to be demonstrated in neurons, vimentin has been shown to have the potential to link the plasma membrane (via binding to the actin-associated protein ankyrin) to the nuclear envelope (via binding to lamin B) (Georgatos and Blobel, 1987, 1988); this property is shared by peripherin (Djabali et al., 1991) as well as the muscle specific intermediate filament desmin (Georgatos et al., 1987). Neurofilament proteins have also been described to contain calcium binding EF-hand-like sequences (Lefebvre and Mushynski, 1988). Though functional roles for these regions is not yet known, one might suppose that they would be involved in a calcium-regulated assembly or post-translational modification process acting either directly on associated proteins or on neurofilament proteins themselves. Some data appear to suggest that NFs can directly bind nucleic acids (Traub et al., 1983 and 1985). Perhaps neurofilaments may serve as general cytoplasmic carrier or reaction surfaces serving to integrate other cytoskeletal networks with the translational machinery and organelles.

Experimental Goals

Despite the prevalence of neurofilament proteins as well as antibodies raised against them, much confusion still exists concerning the role of these proteins in normal as well as disease states. Much of this confusion may be due to a lack

of systematic experimental dissection of the sequence motifs in the unique carboxyterminal tails of NF-M and NF-H. The aim of this study is to inspect these domains in an organized manner using the techniques of molecular biology, bacterial expression and immunochemistry (Harris et al., 1991). Ultimately this project should not only resolve existing discrepancies in the literature due to the use of non-epitope-mapped antibodies, but also facilitate a more precise examination of neurofilament protein interactions and functions in future investigations.

CHAPTER 2
CLONING AND EXPRESSION OF NF TAIL DOMAINS

Introduction

Neurofilaments of the adult mammal are composed predominantly of three proteins, usually referred to as NF-L, NF-M and NF-H, though they may also contain α-internexin, peripherin and vimentin (see Shaw, 1991 for recent review). Despite the fact that the three major proteins have been extensively characterized, we still do not understand many basic features of neurofilament biology. For instance, we know little about how neurofilaments interact with other neural components, virtually nothing about how they are transported in the processes of neurons, how they are organized into bundles or why they become perturbed in a variety of disease states. It is widely assumed that the "effector" regions of intermediate filaments are in the hypervariable C-terminal tail regions (e.g., Traub, 1985; Steinert and Roop, 1988). We have therefore carefully examined the amino-acid sequences of the tail regions of NF-M and NF-H as a first step in elucidating the function of neurofilament tails. Our analysis has allowed us to suggest a nomenclature for the several distinct types of sequence found in these tails (Shaw, 1989, 1991; see Figure 1-1), and we have now extended this analysis

to cover α-internexin, which clearly belongs to the same protein family as NF-L, NF-M and NF-H (Fleigner et al., 1990).

In line with previous studies, we call the region immediately C-terminal to α-helical coil domain "Tail A" (Geisler et al., 1983). C-terminal to this region α-internexin, NF-L, NF-M and NF-H each have a glutamic-acid-rich segment which we call the E-segment (E1 in NF-M). This represents the entire C-terminus in NF-L, but both NF-M and NF-H follow this with short repeated peptides containing the sequence lysine-serine-proline, which we call the KSP segments. In rat NF-H there are nearly 60 KSP and related peptides arranged in an almost unbroken sequence, whereas rat NF-M has 2 at each of 2 positions (KSP1 and KSP2). In NF-M these two KSP sequences are separated by a second glutamic-acid-rich region, E2. The numbers of KSP sequences and the sequence of surrounding amino acids are quite variable across species boundaries; human NF-M, in contrast to rat, has only one KSP at the KSP1 position and 12 at the KSP2 position (Myers et al., 1987), and human NF-H also has a different number and arrangement of KSP sequences when compared to rat NF-H (Lees et al., 1988). The KSP segments are of particular interest since they are major in vivo phosphorylation sites on neurofilaments (Geisler et al., 1987; Lee et al., 1988a). C-terminal to the KSP2 region in NF-M are the SP sequences, which are also in vivo phosphorylation sites, may be present in variable number in different species and are clearly

related to the KSP sequences (Geisler et al., 1987; Shaw, 1989; Xu et al., 1989). Rat NF-M has only one of these sequences, as do human and mouse, although pig and chicken have multiple SP repeats. At the extreme C-terminus of NF-M is a region rich in lysine and glutamic acid which we have called the KE segment. The primary sequence of this region is highly conserved across species boundaries and contains interesting 15 amino-acid repeated sequences (shown as black bars in Figure 1-1; also see Shaw, 1989). We have previously focused attention on the KE segment as potentially a functionally significant region, a speculation strengthened by the finding that the extreme C-terminus of the newly recognized member of the neurofilament subunit protein family, α-internexin, is highly homologous to the extreme C-terminal KE segment of NF-M, and contains a 15 amino-acid peptide sequence very closely related to the NF-M KE repeats (Fleigner et al., 1990). Finally, the analogous region at the extreme C-terminus of NF-H is rich in lysine, glutamic acid and proline, and we have therefore named it the KEP segment.

It seems obvious that these distinct types of sequence must each have different functions. A possible method to find out more about the functions of these regions is to obtain neurofilament protein fragments containing only one of these different types of sequence, although this has not been easy to achieve using standard biochemical methodologies. Recently it has become possible to generate recombinant fusion proteins

containing defined parts of the sequences of larger proteins, a powerful method to produce desired regions of molecules in bulk. We report here on the production of a preliminary panel of fusion proteins containing NF-M and NF-H tail sequences.

Methods

Construction of Fusion Proteins

The fusion proteins are named systematically. In each case the first letter indicates species, R for rat and C for chicken. The second letter indicates the particular neurofilament protein (H for NF-H and M for NF-M), and the two numbers describe the first and last amino acid of the neurofilament sequence included in the fusion protein. Rat NF-M fusion proteins were constructed from the full-length rat cDNA obtained from Dr. Ron Liem, and we use the amino-acid numbering system in the publication describing this clone (Napolitano et al., 1987). Rat NF-H fusion proteins were constructed from the partial cDNA isolated by Dr. Ivan Lieberburg and coworkers (Lieberburg et al., 1989). The sequence of a full-length rat NF-H cDNA has recently been described (Chin and Liem, 1990), and we use the numbering system in this paper. The first amino acid of the Lieberburg clone aligns with amino acid 559 of the full-length Chin and Liem sequence. We also obtained a partial cDNA for chicken NF-M described by Zopf et al. (1987). The numbering of this clone is as defined in the full-length sequence later published by

the same group (Zopf et al., 1990). For fusion protein expression we used various members of the pATH plasmid family (Koerner et al., 1991). These prokaryotic vectors produce the first 324 amino acids of the E. coli enzyme trp-E, a short amino-acid sequence defined by a nucleic acid polylinker and the protein coded for by the cDNA inserted. If the insert does not contain a stop codon a short amino-acid sequence derived from the vector follows the insert sequence, but this sequence is never more than a few amino acids since pATH vectors contains stop codons in all three reading frames immediately following the polylinker. The neurofilament regions included in the rat-derived fusion proteins are shown diagrammatically in Figure 2-1.

RM:677-845. The rat NF-M cDNA was cleaved with Sau3AI, and the 511 base pair fragment corresponding to the KE segment was identified and ligated into BamHI cut pATH 1. The protein coded by this segment starts a few amino acids after the sequence DKKKAESP (the rat SP segment) and includes the entire remaining carboxyterminus of the molecule, a total of 169 amino acids, corresponding to what we have defined as the KE segment. This clone was used to generate the two shorter fusion proteins RM:677-761 and RM:677-732 by chemical cleavage as described below.

RM:549-845. The full-length rat NF-M cDNA was cut with EcoRI and XhoI to produce a 1064 base pair fragment with an XhoI site on the 5′ and an EcoRI site on the 3′ end. The

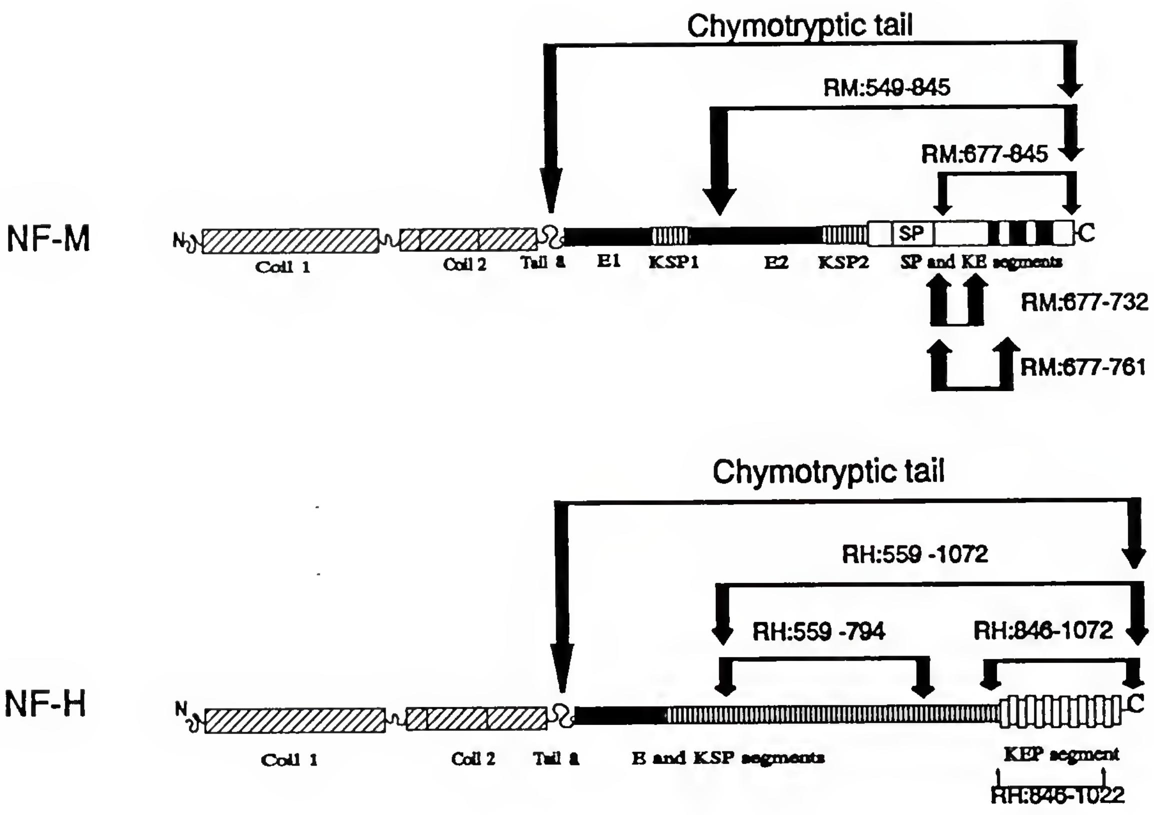

Figure 2-1. Map of rat NF-M and NF-H fusion proteins and proteolytic fragments.

protein coded for by this insert includes the KE, the SP, KSP2 and part of the E2 sequences. This was ligated into pATH23 that had been digested with SalI and EcoRI.

CM:381-605. This was derived from the partial cDNA of Zopf et al., (1987). A 673 base pair fragment was cut out of this clone with Sau3AI corresponding to bases 94 to 766. This codes for the last segment of the α-helical rod domain, the "tail A" region, the chicken E1 and E2 segments and the 4 NF-M KSP sequences at KSP2, accounting for 225 amino acids (the NF-M KSP1 segment is less evolutionarily conserved than the NF-M KSP2 segment and is missing from chicken). The clone ends between the KSP sequences and before the start of the multiply repeated chicken SP sequences. The 673 base pair cDNA fragment was gel purified and ligated into BamHI treated pATH 2.

RH:559-1072. We isolated three individual 2.2 kilobase cDNA clones coding for rat NF-H from a commercial bacteriophage lambda gt-11 rat brain cDNA library (Clontech) by screening nitrocellulose filter replicas with a mixture of polyclonal antibodies directed against all three neurofilament triplet proteins. The ß-galactosidase fusion proteins expressed by these clones reacted very strongly with N52, a phosphate independent NF-H monoclonal antibody. Further analysis showed that two of these cDNAs were, somewhat surprisingly, absolutely identical in size, restriction digest pattern and initial 5′ and 3′ sequence to the rat 2168 base-pair NF-H clone described by Lieberburg et al. (1989). There

is no EcoRI site within the full-length rat cDNA clone, and we did not use the same cDNA library as the Lieberburg group. Presumably this finding reflects either specific nucleic acid cleavage or blockage of polymerase during or possibly prior to library construction. We decided not to use our own clones since this would have required costly and time-consuming full nucleic acid sequencing. Furthermore, a pATH construct containing the full-length Lieberburg clone had already been produced and was kindly donated by Dr. Ron Liem (Braxton et al., 1989). These authors excised the Lieberburg NF-H cDNA using EcoRI and ligated it into EcoRI-digested pATH 1.

RH:846-1072. The Lieberburg rat NF-H cDNA was cleaved with Sau3AI and EcoRI to produce a 1272 base pair fragment which corresponds to the KEP segment of NF-H preceded by the last four irregular NF-H KSP sequences of the KSP repeats. This was ligated into pATH 23 following treatment of the vector with BamHI and EcoRI.

RH:559-794. The first 704 b.p. of the rat NF-H cDNA of Lieberburg et al. (1989) was excised using EcoRI and Sau3AI. This corresponds to the most perfect tandem KSP repeats towards the amino terminus of the rat NF-H tail, containing 37 repeats with the consensus AKSPAE. The appropriate cDNA was ligated into EcoRI/BamHI cut pATH 20.

Isolation and Verification of Fusion Protein Clones

Transfected HB101 E. coli were grown on ampicillin plates, and 15-20 colonies were selected for screening. Cultures were grown overnight in 5ml of M9 media plus 50μg/ml ampicillin and 20μg/ml tryptophan. These cultures were diluted 1:100 in fresh M9 media with no added tryptophan but with ampicillin as before. Twenty μg/ml indoleacrylic acid (IAA) was added to induce fusion protein expression. After overnight growth at 37°C cells were pelleted and dissolved in SDS-PAGE sample buffer and run out on 7.5% gels. Clones which produced a large extra protein band at about the molecular weight expected were selected for further study (see Figure 2-2A for typical example). Immunoblots from such clones were tested with polyclonal and monoclonal antibodies against the appropriate protein, and those producing convincing positive reactions at least with polyclonal antibodies were grown up to prepare plasmid DNA. The correct insert size was then verified electrophoretically following cleavage with an appropriate restriction endonuclease. Alternately, insert size was rapidly verified using the polymerase chain reaction (PCR) with probes recognizing trp-E and polylinker sequences (i.e., on either side of the insert; see Figure 2-2B). The 5′ primer was AGCCGCCAGATTGAGATC and the 3′ primer was TAATTCTCATGTTTGACAGCT; both primers will work on all current

members of the pATH family. We also ran out fractionated fusion protein (see below) on SDS-PAGE and transferred to PVDF for amino-acid analysis; in every case we obtained amino-acid profiles extremely close to the expected values. Finally, RM:677-845 and RM:549-845 were directly sequenced using the United States Biochemicals Sequenase kit following the manufacturers instructions for double stranded DNA. We used the two oligonucleotide primers described above and found the 3′ and 5′ ends of the constructs to be exactly as predicted.

Fusion Protein Expression

Fusion protein expression was induced in 500ml cultures by addition of 10-20μg/ml IAA following withdrawal of tryptophan (see Koerner et al., 1991). Cultures were grown for up to 36 hours to obtain maximum fusion protein yield. Fusion proteins were harvested by the lysozyme lysis inclusion body procedure described in Ausubel et al. (1990). Inclusion body material was dissolved in freshly deionized 6M urea, 10mM sodium phosphate, 1mM PMSF, and 1mM EDTA pH=7.5 and subjected to ion exchange chromatography on DEAE-cellulose as described previously (Shaw and Hawkins, 1992). Proteins were eluted with a NaCl gradient from 0.0M to 0.25M and fractions were examined by SDS-PAGE (see Figure 2-3). The cleanest fusion protein-containing fractions were extensively dialyzed against phosphate buffered saline containing 1mM PMSF and were used for further experiments and antibody production.

Chemical Cleavage of Fusion Proteins

Hydroxylamine under basic conditions cleaves polypeptide chains at asparagine-glycine (NG) sequences (Bornstein and Balian, 1977; Moks et al., 1987). There are two convenient NG sequences in the KE segment of NF-M, although no other part of the NF-M or NF-H tails contains these sequences. Appropriate preparations of neurofilaments or fusion proteins were taken up in hydroxylamine cleavage solution (2M hydroxylamine, 0.2M Tris pH=9.00) and incubated at 37°C for 1 hour. The reaction was stopped by neutralization with 1M HCl, and cleaved proteins were mixed with gel sample buffer and run out on SDS-PAGE for immunoblotting.

The last 50 amino acids of the rat NF-H tail could be clipped off using cyanogen bromide (CNBr). The RH:846-1072 fusion protein was dialyzed against PBS, concentrated and then cleaved by CNBr by adding 190 µl fusion protein (approximately 0.5 mg/ml) and 190 mg CNBr (Sigma) to 760 µl of 88% formic acid. This mixture was mixed in a sealed tube and left overnight at ambient temperature in the fume hood. The sample was then aliquoted into 10 tubes and dried down in a vortex evaporator at 60°C for at least 6 hours. Aliquots were sealed and stored at -20°C until use.

Results

We transfected bacteria with our nucleic acid constructs and grew up minicultures of individual ampicillin-resistant colonies under conditions designed to induce fusion protein expression. After overnight growth bacteria were pelleted and analyzed by SDS-PAGE. Figure 2-2A shows the result of screening 11 colonies which had been transfected with the plasmid designed to produce the RH:559-794 fusion protein. Lanes 1-9 show colonies all of which produce a very prominent protein band missing in lane 12, which was transfected with vector lacking a neurofilament cDNA insert. Lanes 10 and 11 show bacterial colonies which failed to express the desired insert. We verified the identity of these clones using several methods. All of the fusion proteins could be stained with appropriate polyclonal antibodies as well as certain monoclonal antibodies to neurofilaments. We checked the size of the cDNA insert using restriction mapping or the polymerase chain reaction (Figure 2-2B). The inserts were of the expected size in every case. Fusion proteins were purified from bacterial inclusion body preparations following dissolving in phosphate-buffered 6M urea. Ion exchange chromatography on DEAE-cellulose proved to be an efficient method to purify the fusion proteins (Figure 2-3).

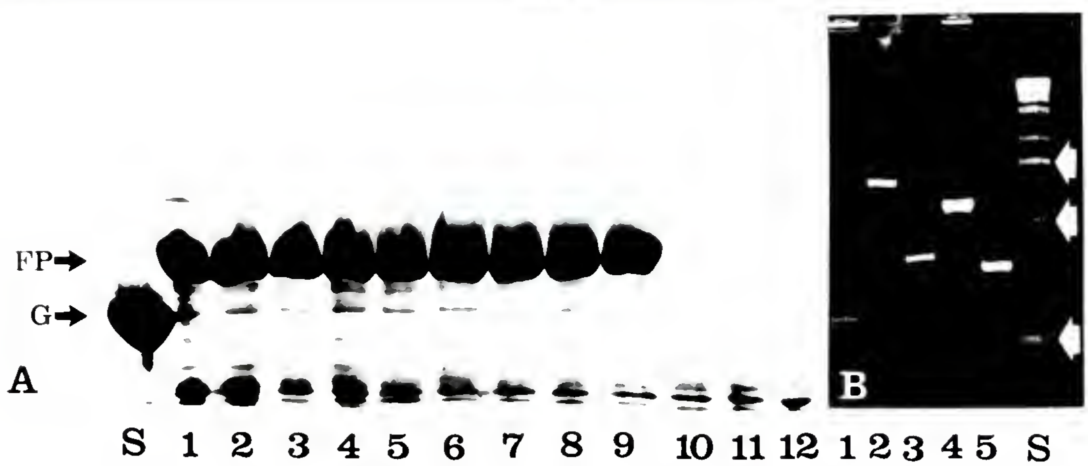

Figure 2-2. Construct screening methods. A: Coomassie Brilliant Blue stained SDS-PA gel showing a screening for fusion protein expression by RH:559-794 clones. Lane S is a standard containing GFAP (G, 50 kDa). Lanes 1-9 contain a major band (FP) corresponding to the fusion protein which is absent in the control (bacteria transfected with empty pATH vector) in lane 12. Lanes 10 and 11 are Ampicillin-resistant colonies that did not incorporate the correct insert.
B: Ethidium bromide stained agarose gel of PCR products derived from RM:677-845, RH:846-1072, RH:559-794, RM:549-845 and CM:381-605 (lanes 1-5, respectively). The three arrowed standards (lane S) are, bottom to top, 506, 1018 and 1636 bp. Calculated PCR product sizes are 618, 1367, 788, 1158 and 762 bp respectively, in excellent agreement with the gel.

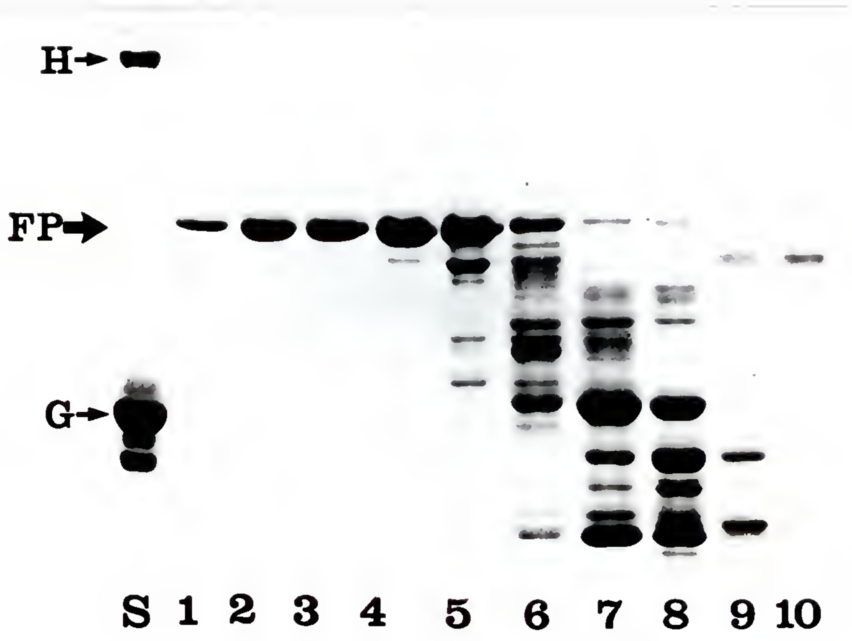

Figure 2-3. Coomassie Brilliant Blue stained SDS-PA gel showing a DEAE-cellulose purification of RH:846-1072. Lane S is a standard containing bovine GFAP (G, 50 kDa) and NF-H (H, 220kDa). Lanes 1-10 are sequential fractions produced by the 0.0 M to 0.25 M NaCl gradient. The fusion protein (FP) is eluted cleanly before most of the bacterial contaminants.

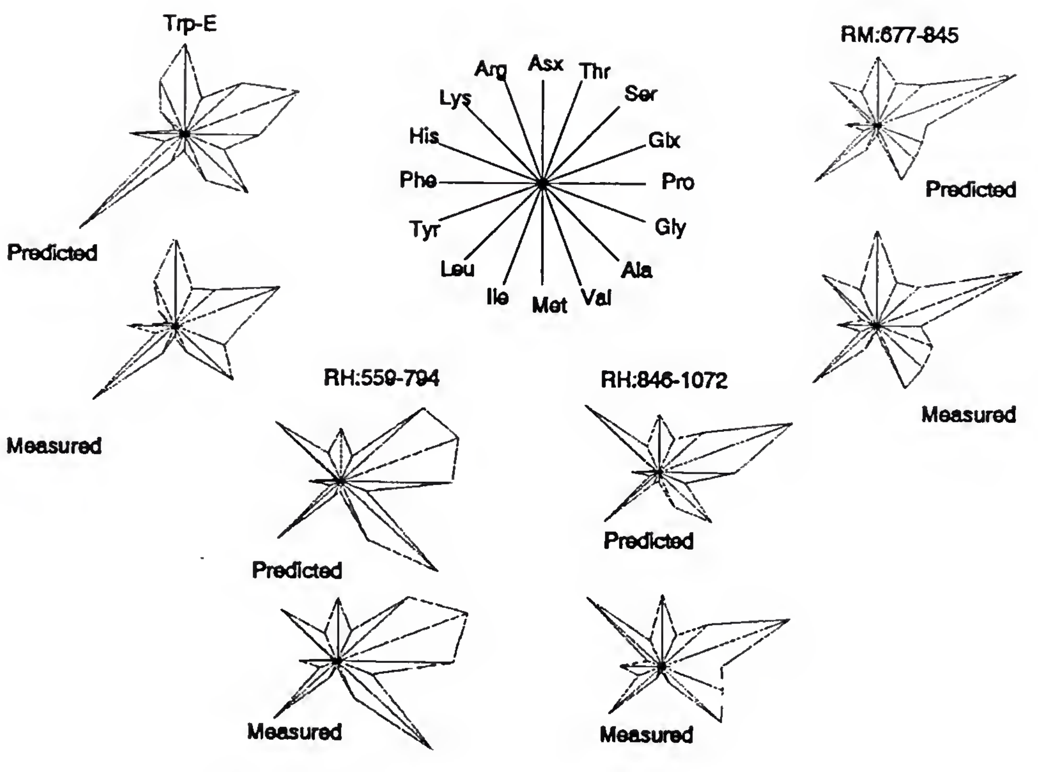

Figure 2-4. Plots depicting predicted versus measured amino acid compositions for trpE alone, RM:677-845, RH:559-794 and RH:846-1072. A reference wheel shows the direction in which the relative percentage of each amino acid is plotted.

The fusion proteins were transferred to PVDF membranes and processed for amino-acid analysis and in every case gave profiles very close to that predicted (Figure 2-4). We have also verified the nucleic acid sequence of RM:677-845 by direct nucleic acid sequencing and found it to be absolutely as expected. In many cases we obtained multiple clones, as in the case shown in Figure 2-2A, which produced identical protein profiles.

Table 2-1. Real and Apparent SDS-PAGE Molecular Weights of Fusion Proteins:

Fusion protein	# Amino Acids	Calculated MW (kDa)	SDS-PAGE MW (kDa)
trpE	336	37.3	37
RM:677-845	503	55.7	68
RM:677-761	419	46.5	55
RM:677-732	390	43.3	48.5
RM:549-845	633	70.5	82.2
CM:381-605	550	60.5	75
RH:559-1072	833	91.5	133
RH:559-794	566	60.3	71.3
RH:846-1072	567	63	76.2
TD-RH:846-1072	411	45	59
TDCNBr-RH:846-1022	216	24	50

We measured the SDS-PAGE molecular weights of the fusion proteins as well as the proteolytic fragments of RM:677-845 and RH:846-1072 and found that, although trp-E alone ran very close to predicted from its molecular weight, in nearly every case the fusion protein ran more slowly (see Table 2-1). We conclude that all parts of the NF-M and NF-H tails exhibit this unusual SDS-PAGE mobility. We calculated the contribution to SDS-PAGE mobility due to each neurofilament insert and

compared that to the known molecular weight. In every case the insert ran between 1.4 and 1.8 times slower than expected from its molecular weight.

Discussion

Six cDNA constructs containing overlapping but distinct tail domains for NF-M and NF-H were successfully cloned and expressed in bacteria. All constructs were verified by PCR amplification of a cDNA insert of an appropriate size. Some cDNA constructs were at least partially sequenced to verify the ligation sites. Fusion proteins were checked for appropriate molecular weights, amino-acid compositions and positive immunostaining on western blots using existing neurofilament polyclonal antibodies. Finally, since all fusion proteins exhibit the unusual electrophoretic properties of the parent molecules (discussed below) and since all of them, when injected into rabbits and mice, generate neurofilament antibodies (see Chapter 3), there can be little doubt about the correctness of the constructs.

Rat NF-H and NF-M run at about 200kDa and 145kDa, although the real molecular weights are 115.3kDa and 95.6kDa as determined from the full amino-acid sequence (Chin and Liem, 1990; Napolitano et al., 1987). Previous studies have shown that the tail sequences of NF-M and NF-H are responsible for this unusual gel mobility (Kaufmann et al., 1984). Here we show that this unusual property is distributed throughout the

entire tail segment. The common features of the NF-M and NF-H tail sequences are the tail A sequences, the KSP repeats, the glutamic-acid-rich E regions and the overall high charge density. Our results suggest that the high charge density is much more important for slowing down these proteins in SDS-PAGE, since all neurofilament tail fusion proteins, irrespective of their content, run unusually slowly on SDS-PAGE. We also note that the chymotryptic tails seem to run relatively even slower on SDS-PAGE than any of our fusion proteins, suggesting that either the intact tail somehow interacts with the gel matrix in a manner different from the fusion proteins, or that phosphate groups which are resistant to enzymatic dephosphorylation also contribute to SDS-PAGE gel retardation in the native molecules.

The recombinant fusion proteins described here can be used not only to epitope-map existing neurofilament antibodies, but also to generate antibodies against targeted domains which may be underrepresented by existing markers. These constructs are also of great potential use in biochemical and cell biology studies of neurofilament dynamics, phosphorylation and function.

CHAPTER 3
PRODUCTION AND CHARACTERIZATION OF NF FUSION PROTEIN ANTIBODIES

Introduction

In spite of there being a considerable number of NF-M and NF-H antibodies available both commercially (e.g. - Sigma and Boeringher Mannheim) and from large laboratories doing intermediate filament research (e.g. - Weber and Osborn, Lee and Trojanowski), nearly none of these antibodies have been previously characterized beyond their ability to stain the rod, chymotryptic tail or phosphorylated KSP repeats. The majority of neurofilament antibodies react with the highly charged, phosphorylated KSP epitopes, which are shared with tau and MAP2. Cross-reactivity between antibodies made against high molecular weight NFs (NF-M and NF-H) and MAPs (tau and MAP2) has caused considerable confusion in immunochemical studies on degenerating neurons, like those of Alzheimer's disease. In addition, the chymotryptic tails of NF-M and NF-H have been difficult to further dissect protein chemically because of the large number of repeated elements and lack of convenient cleavage sites. With the panel of fusion proteins and fragments we have generated, our laboratory was able to refine the epitope maps of existing

NF-M and NF-H monoclonal antibodies. After screening over 300 existing monoclonals, we have been able to further map 22 of them; the majority of these antibodies (the RMO series) were made by Dr. Virginia Lee and co-workers. By using fusion proteins containing regions other than the KSP repeats, we have also been able to make new antibodies that recognize unique epitopes in both NF-M and NF-H.

Methods

Available NF Antibodies

NR4, NN18, NE14 and N52 are commercially available from several vendors (including Boerhinger-Mannheim and Sigma). These and the other N series antibodies have been partially characterized in a series of previous publications (Debus et al., 1983, Shaw et al., 1984, 1986). The RMO series (#1-310) of NF-M and NF-H monoclonals were made available by Dr. Virginia Lee. SMI31, SMI32, SMI33 and SMI34 can be obtained from Sternberger-Meyer Immunocytochemicals. RT97 and 8D8 were the kind gift of Dr. Brian Anderton. The α-IFA clone was obtained from the American Type Culture Collection and used to produce antibody rich hybridoma supernatents (Pruss et al., 1981). The polyclonal antibodies (H301, H298, GP64 and GP63) were made against purified rat neurofilament subunits isolated from sciatic nerve (Shaw et al., 1981). Neurofilament antibodies were used at empirically determined concentrations

which were the minimum required to give saturated staining of NFs in frozen sections of rat brain.

Western Blotting

Fusion proteins purified by DEAE-cellulose chromatography and their proteolytic fragments were run out on either 4-20% gradient (BioRad) or constant percentage (6% or 10%) combless SDS polyacrylamide gels and transferred to nitrocellulose filters using standard methodologies. Filters were cut into strips and probed with various antibodies. Second antibodies were appropriate goat anti-rabbit, goat anti-guinea pig and goat anti-mouse antisera coupled to alkaline phosphatase. Colored reaction product was generated using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (second antibodies and chromagens obtained from Sigma).

Production of Fusion Protein Antibodies

Fusion proteins purified as described above were injected at about 200µg per animal into rabbits and mice using standard methodologies. All animals injected so far have produced strong anti-neurofilament antibodies after only two injections. One rabbit was injected with each of the purified fusion proteins RM:677-845, RH:559-794 and RH:846-1072. The antisera resulting from these are referred to as anti-NF-M KE, anti-NF-H KSP and anti-NF-H KEP respectively. Rabbit antibodies were characterized by immunoblotting and on frozen

sections of rat brain and spinal cord. We have raised monoclonal antibodies against the RM:677-845 and RH:846-1072 fusion proteins. Mice were injected following a similar protocol to the rabbits. Hybridomas were screened on ELISA using bovine NF-M or rat NF-H, and positive clones were retested firstly by immunofluorescence on frozen sections of rat brain and then by immunoblotting on the appropriate fusion protein and porcine neurofilament protein. Clones of interest were subcloned twice by limiting dilutions in 96 well dishes on a monolayer of 3T3 cells.

Results

We used purified fusion proteins to screen our large panel of neurofilament antibodies (see Figure 3-1 for typical results). We have previously shown that all of the N series antibodies stain only the chymotryptic tails of NF-M and NF-H, and that most of these antibodies show reduced or no staining following enzymatic dephosphorylation of neurofilament proteins (Shaw et al., 1986). The antibodies which do not recognize enzymatically dephosphorylated neurofilaments fail to stain our fusion proteins. NE14, NF1, RT97, SMI31 and SMI34 fall into this family of antibodies, in line with previous suggestions that these antibodies recognize predominantly phosphorylated KSP sequences (Lee et al., 1988a). Three of the antibodies tested (SMI32, SMI33 and N52) showed very strong staining of the RH:559-1072 and RH:559-794 clones both of

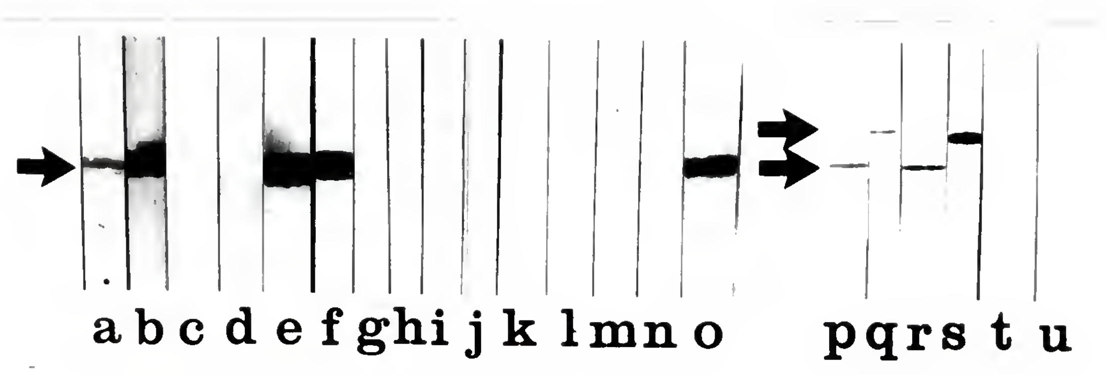

Figure 3-1. Selected results with RH:559-794, RH:846-1072 and RM:677-845. Lanes a-o are blots of RH:559-794 (left arrow). Lane a=H298 (rabbit anti-NF-M); b=H301 (rabbit anti-NF-H); c=NN18; d=SMI34; e=SMI33; f=SMI32; g=SMI31; h=8D8; i=RT97; j=NE14; k=NF1; l=NC43; m=NL34; n=NA34; o=N52. Lanes p-u are blots of a mixture of purified RH:846-1072 (upper arrow) and RM:677-845 (lower arrow). Lane p=GP63 (guinea pig anti-NF-M); q=GP64 (guinea pig anti-NF-H); r=H298; s=H301; t=NN18; u=N52.

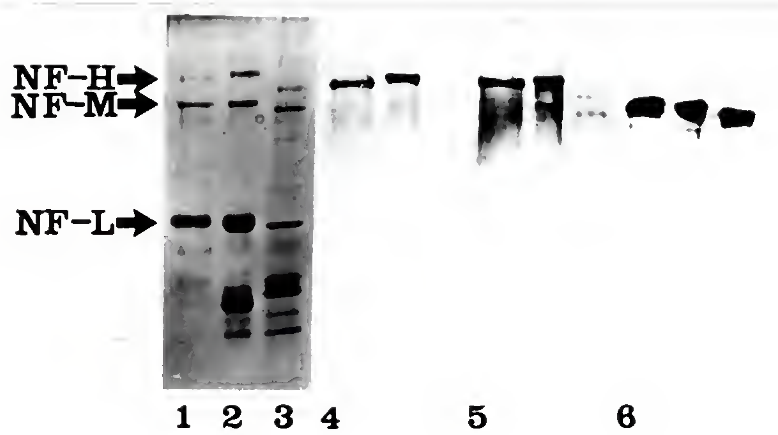

Figure 3-2. Preliminary cross-species characterization of rabbit polyclonal anti-rat NF-H KEP, NF-H KSP and NF-M KE. Lanes 1-3 are Coomassie Brilliant Blue stained preparation of neurofilament from pig, cow and chicken respectively. Arrows indicate the positions of porcine NF-(H), NF-(M) and NF-(L). Lanes 4-6 are corresponding immunoblots reacted with anti-NF-H KEP, anti-NF-H KSP and anti-NF-M KE. respectively.

which contain multiple KSP repeats (Figure 3-1, lanes e, f and o). These three antibodies, however, gave divergent results on the other fusion proteins. N52 failed to stain either of the fusion proteins containing NF-M-type KSP sequences (RM:549-845 and CM:381-605), suggesting that it is specific for some feature of the NF-H KSP motif. N52 showed weak, but definitely positive, staining of RH:846-1072, which contains the last four irregular rat NF-H KSP sequences (Figure 3-1, lane u). N52 therefore does not absolutely require either large numbers or highly regular NF-H type KSP repeats. SMI32 showed weak staining of RM:549-845, which contains the rat NF-M KSP2 sequence, did not stain the chicken fusion protein and gave very weak and equivocal staining of RH:846-1072. We conclude that SMI32 is somewhat less specific for NF-H KSP sequences than N52, but that it may have more rigid requirements in terms of the length or organization of those sequences. SMI33 gave the simplest pattern; it stained all fusion proteins which contain typical neurofilament KSP sequences irrespective of whether they were in NF-M or NF-H, with one exception. RM:677-845 actually contains one KSP sequence (GDKSPQE, 717-723). However this is not evolutionarily conserved, is not preceded by neutral or hydrophobic amino acids and is apparently not an in vivo phosphorylation site (Xu et al., 1989). It seems that this tripeptide arose fortuitously and is probably not related to the other KSP sequences. NN18, a phosphorylation-independent monoclonal antibody reactive with

the chymotryptic tail of NF-M stains the RM:677-845 and RM:549-845 fusion proteins strongly and specifically, localizing the NN18 epitope to somewhere with in the NF-M KE segment (Figure 3-1, lane t, for higher resolution mapping see below). Anti-IFA, a monoclonal antibody which recognizes a determinant in the α-helical rod region of all intermediate filaments (Pruss et al., 1981) stains only the CM:381-605 fusion protein. This finding is completely in agreement with the results of Geisler et al. (1983), who localized the anti-IFA epitope to the extreme C-terminus of the α-helical rod region, which is included only in CM:381-605. Finally we previously described NL34, NA34 and NM46 as antibodies which recognize NF-H and/or NF-M tails, but which are partially or totally phosphate-independent (Shaw et al., 1986). Two of them failed to stain any of the fusion proteins and the third, NA34, showed weak staining for the fusion proteins containing NF-H KSP sequences (e.g. Figure 3-1, lane n).

We have raised antibodies against the rat fusion proteins RM:677-845, RH:559-794 and RH:846-1072. We refer to these antibodies as anti-NF-M KE, anti-NF-H KSP and anti-NF-H KEP respectively. All three antisera produce high-titre neurofilament staining on frozen sections of rat brain and strongly and specifically recognize the appropriate immunogen and intact rat neurofilament subunit on immunoblots (not shown). We examined the ability of these antibodies to recognize neurofilament subunits in cow, pig and chicken.

Anti-NF-H KEP recognized pig and cow NF-H strongly on immunoblots, but gave a very weak signal on chicken NF-H (Figure 3-2, lane 4). Anti-NF-H KSP proved to stain all mammalian NF-H bands very strongly, but also showed weaker staining for mammalian NF-M, except in the case of bovine NF-M, which was stained much more strongly than rat or pig (Figure 3-2, lane 5). Anti-NF-H KSP weakly recognized both chicken NF-H and chicken NF-M. In contrast the anti-NF-M KE strongly recognized NF-M in all species tried and showed no reactivity for NF-H (Figure 3-2, lane 6). All three antibodies recognized axonal neurofilaments in frozen sections of rat brain very strongly and specifically. In line with the immunoblot results all three antibodies gave clearly neurofilamentous staining on frozen sections of chicken brain, but anti-NF-M KE stained very strongly in contrast to anti-NF-H KSP and anti-NF-H KEP which were much weaker (not shown).

We have also raised monoclonal antibodies against the NF-M KE (RM:677-845) and NF-H KEP (RH:846-1072) segments. We injected mice and made hybridomas using standard procedures, and screened supernatents on either native pig NF-M, rat NF-H or the fusion proteins. Many hybridomas recognized native NFs in ELISA assays, and from these we subcloned eight NF-M KE and 2 NF-H KEP monoclonals which stained neurofilaments strongly in frozen sections of rat brain and also recognized only the appropriate native NF subunit strongly on immunoblots. We also isolated several hybridomas which stain the trp-E carrier

protein, one of which, called 4A10, we subcloned and partially characterized. In order to further localize the epitopes for these antibodies, we cleaved the RM:677-845 fusion protein using hydroxylamine, a reagent that cuts between asparagine-glycine (NG) residues under basic conditions (Bornstein and Balian, 1977), and the RH:846-1072 fusion protein with cyanogen bromide.

The extreme C-terminus of rat NF-M contains two conserved NG sequences, at 732-733 and 761-762. Complete cleavage at these sites should therefore release an 84 amino acid C-terminal fragment and a 29 amino acid fragment immediately preterminal to this. Both of these sites are within the RM:677-845 clone and complete NG cleavage should therefore produce three fusion protein fragments which contain neurofilament sequences corresponding to 677-732, 733-761 and 762-845. The N-terminal 677-732 fragment should remain attached to the trp-E carrier protein. We performed the NG cleavage as described above and ran the product on SDS-PAGE for protein visualization (Figure 3-3, lanes U and C). We noted three major protein bands in the SDS-PAGE molecular weight range 50-70kDa, which made it clear that the cleavage was only partial under our conditions. The highest band comigrated with the RM:677-845 fusion protein and was therefore presumably uncleaved protein. We assumed that the lower bands represented RM:677-845 cleaved at one or other of the two NG sites. To test this we transferred all three

polypeptides to PVDF membranes and obtained amino-acid composition data. The profiles obtained (not shown) were consistent with this interpretation, and we therefore refer to the three bands in Figure 3-3 as RM:677-845, RM:677-761 and RM:677-732. These three large protein bands could be conveniently used for immunoblotting and epitope localization experiments with our panel of monoclonal antibodies (the released 29 and 84 amino acid C-terminal fragments are rather small and much less easy to immunoblot under our standard conditions). The results were quite clear; NN18, RMO1 and RMO59, stain all three bands, two of our new monoclonals stain the two larger bands and the remaining 6 stain only the largest band. In addition many of the RMO monoclonal antibodies, including the better described RMO255 and RMO54, also only stain the largest band. Dr. Lee has localized the epitope for RMO255 to the last 20 amino acids of NF-M using a peptide containing those last 20 amino acids in a competitive ELISA assay (personal communication). Finally, and as expected, our trp-E antibody stains all three bands. These results firmly map the epitopes for the 11 antibodies as shown in Figure 3-5. We always saw staining for a fourth band just below RM:677-732 with an apparent molecular weight of about 44kDa (labelled T in Figure 3-3), which is a minor component biochemically. We conclude that this protein is produced by cleavage within the trp-E molecule, by removal of about 200 amino acids. In line with this interpretation the preterminal

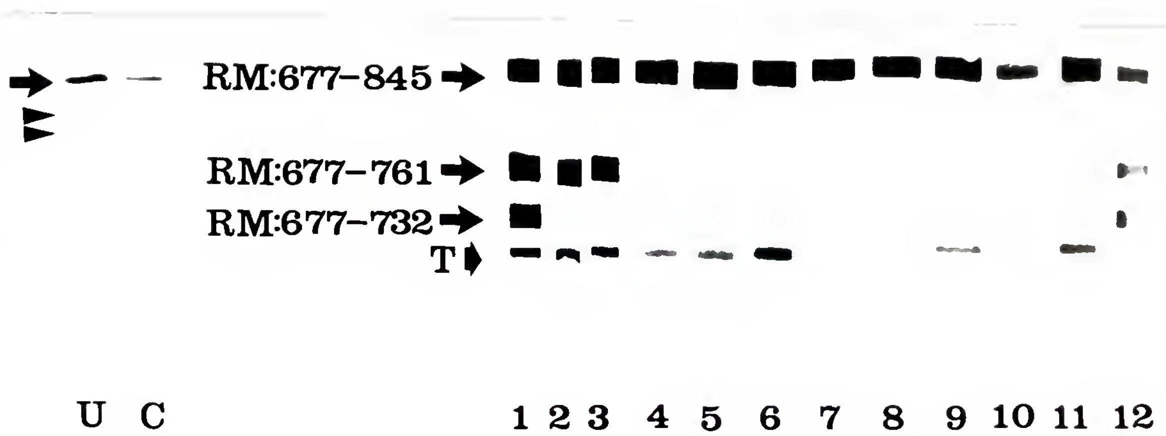

Figure 3-3. Production and use of hydroxylamine cleaved RM:677-845 to map antibodies to NF-M C-terminal. Lanes U and C show Coomassie Brilliant Blue stained gels of uncleaved (U) and cleaved (C) RM:677-845 fusion protein isolated by ion exchange chromatography in 6M urea as described. Note partial cleavage, so that a band of apparent molecular weight 66kDa is seen in both lanes. Note also the presence of two lower molecular weight fragments in C which run at 55kDa and 48.5kDa. Lanes 1 to 12 are immunoblots from 7.5% acrylamide curtain gels of preparations identical to lane C. The antibodies are 1=NN18, 2=5C6, 3=IG12, 4=5G9, 5=IG9, 6=4H4, 7=5E7, 8=5B12, 9=RMO54, 10=RMO255, 11=3H11 and 12=4A10. The position of the three fusion proteins is as indicated. T denotes a fragment produced by N-terminal cleavage of trp-E.

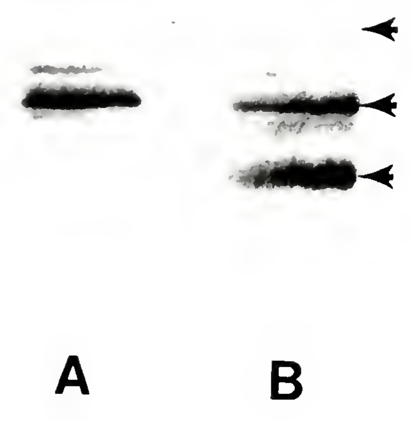

Figure 3-4. Immunoblots of cyanogen bromide (CNBr) cleaved RH:846-1072 to map NF-H KEP monoclonals 3G3 and 5B8. Lane A is stained with 3G3 and lane B with 5B8. Arrowheads mark the positions of the uncleaved fusion protein RH:846-1072 (top), a degradation product of RH:846-1072 missing part of the trp-E head, TD-RH:846-1072 (middle), and the CNBr cleavage fragment of TD-RH:846-1022, TDCNBr-RH:846-1022 (bottom). No protein band was present in the top position in samples treated by CNBr.

antibodies NN18, 5C6 and IG12, but none of the extreme C-terminal antibodies stained a further protein band below T; this is consistent with our conclusion since a fusion protein missing part of the N-terminus should also generate a further similar but lower apparent molecular weight degradation ladder to that shown in Figure 3-3. Upon re-examination several of our other fusion protein preparations contained a second minor protein band running at about 22kDa lower than the major component, also consistent with a cleavage event within the trp-E molecule, probably generated enzymatically either in vivo or during our inclusion-body preparation.

Using cyanogen bromide (which cleaves after methionine) we were also able to produce a fragment of the NF-H KEP fusion protein which is missing the last 50 amino acids (RH:846-1022). Western blots of cyanogen bromide treated RH:846-1072 were stained with our two monoclonal KEP antibodies (Figure 3-4). Both antibodies (3G3 and 5B8) stain the uncleaved fusion protein (RH:846-1072) and its trp-E-degradation product (TD-RH:846-1072), but only 3G3 stained the large fragment (TDCNBr-RH:846-1022). This pattern implies that the epitope of 5B8 may be in the last 50 amino acids, whereas that of 3G3 is likely to be within the preterminal KEP region. It is of note that out of all the antibodies we screened, these two monoclonals are the only antibodies (other than the rabbit polyclonal anti-NF-H KEP) whose epitopes lie solely within the KEP domain of NF-H. Although they contain a degradation

product (TD-RH:846-1072, indicated by the middle arrowhead on Figure 3-4) missing part of the the trp-E head, samples treated with CNBr contain no detectable uncleaved RH:846-1072 (indicated by the position of the top arrowhead in Figure 3-4); the lack of any uncleaved material in the CNBr treated samples is likely due to the presence of 6 methionine cleavage sites in the trp-E head domain.

We tested all newly produced monoclonal antibodies on several of the fusion proteins as was done for the antibodies we already had available. As expected, all of the KE antibodies recognized the RM:549-845, which includes the sequences used as immunogen. None of the KE monoclonals showed the slightest reactivity with the RH:846-1072 fusion protein, which contains the NF-H KEP segment, which can be considered as at least analogous to the NF-M KE segment. Similarly, the KEP monoclonals 3G3 and 5B8 do not stain any of the fusion proteins that do not contain the NF-H KEP segment. We could not do the same experiment in such a straightforward manner with our new polyclonal antibodies, since they all have some recognition for trp-E in addition to the neurofilament sequences. We therefore tried various older polyclonal antisera from animals which were injected with native NF-M or NF-H, but which nonetheless produced antibodies which cross-reacted with the two proteins (Shaw and Weber, 1981). Despite this cross-reactivity the anti-NF-H crude sera were completely specific for the NF-H fusion proteins and the anti-NF-M crude

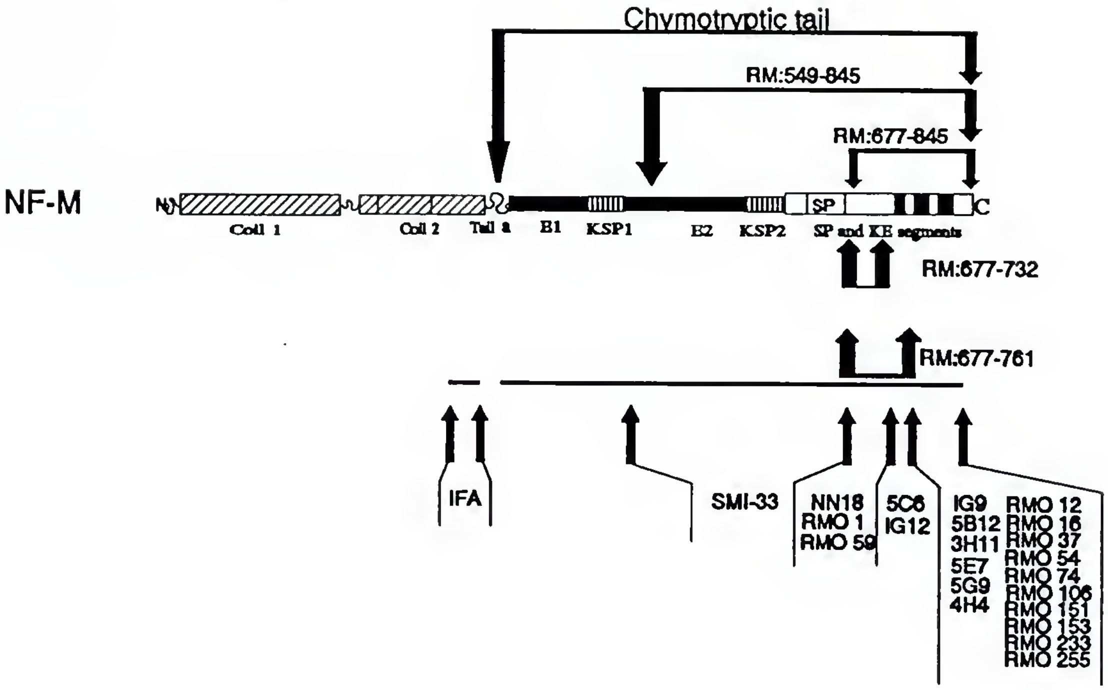

Figure 3-5. Epitope maps of NF-M and NF-H tails. Only antibodies which show strong and unequivocal staining are shown. The epitope of monoclonal IFA has been previously mapped (Geisler et al., 1983).

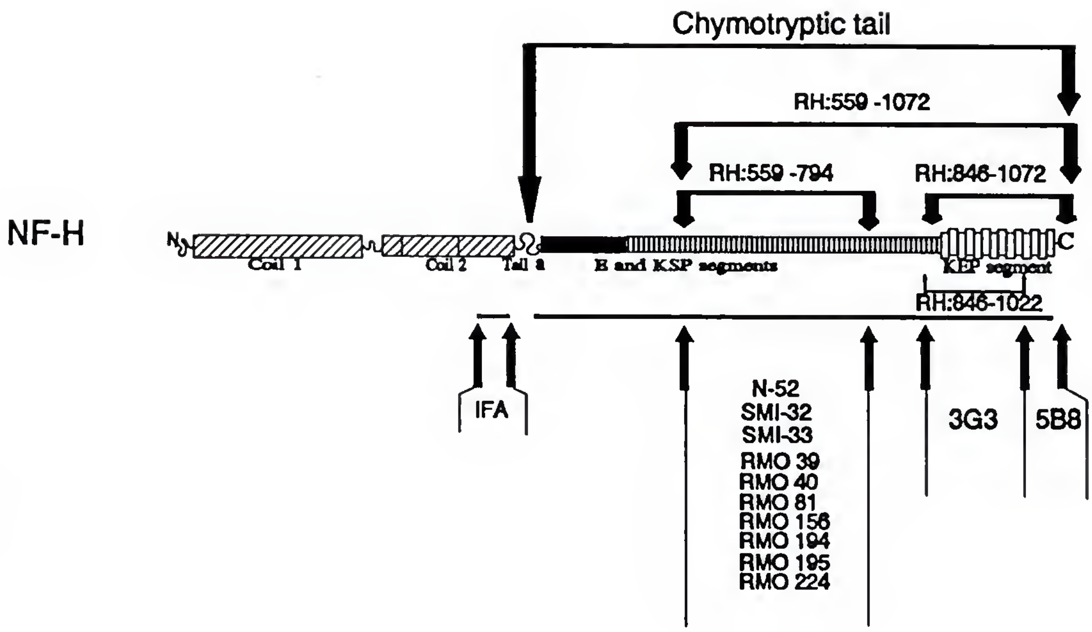

Figure 3-5--continued.

sera were completely specific for the NF-M fusion proteins (Figure 3-1, lanes q-s). Clearly the cross-reactivity exhibited by these antibodies was not due to any immunological similarity between the extreme C-termini of NF-M and NF-H. These NF-M and NF-H polyclonal antibodies showed some degree of recognition for fusion proteins contain KSP sequences, suggesting that KSP sequences are at least partially responsible for the cross-reactivity of these antisera (e.g., Figure 3-1, lanes a,b).

Discussion

Previous data have shown that the majority of currently available neurofilament antibodies recognize phosphorylated KSP sequences in the tails of NF-M and NF-H (e.g. Carden et al., 1985; Shaw et al., 1986; Lee et al., 1988a; Coleman and Anderton, 1990). The immunodominance of the KSP region has caused some confusion; many proteins apart from neurofilaments contain the KSP motif, notably the microtubule associated proteins tau and MAP2, and immunological cross-reactivity between these proteins and NF-M and NF-H has been well documented (Kziesak-Reding et al., 1987; Nukina et al., 1987; Lee et al., 1988b). Clearly antibodies which are known to recognize non-KSP parts of NF-M and NF-H would be very useful for a variety of studies. We now have a growing panel of such antibodies, allowing a more refined interpretation of

many previous studies and the design of better experiments in future.

The detailed characterization of the commercially available NF-H monoclonal antibody N52 has not been previously described, although we have previously shown that it recognizes the native chymotryptic tail of axonal NF-H, and that its binding does not appear to be affected by enzymatic dephosphorylation (Shaw et al., 1986). We now show that N52 belongs to the large class of antibodies that recognize the regions of NF-H which contain Lys-Ser-Pro (KSP) repeated sequences. Given that the antibody recognizes the KSP segments, it is perhaps rather surprising that binding is not affected by the level of NF-H phosphorylation. This antibody also weakly recognizes our RH:846-1072 fusion protein which contains the last four disorganized KSP sequences, suggesting that N52 is relatively insensitive to the exact primary sequence of the NF-H KSP repeats. However N52 does not recognize the NF-M KSP motifs in either of the NF-M clones which contain these sequences, showing that some feature of the NF-H repeats is important for efficient binding. In contrast SMI33, a widely used phosphorylation independent monoclonal antibody, stains all fusion proteins which contain typical KSP sequences, although the recognition of RH:846-1072 was extremely weak, so that staining is not visible on Figure 3-1 (lane g). RM:549-845 contains only two KSP sequences, and CM:381-605 contains four KSP tripeptides within the sequence

VKSPPAKSPPKSPPKSPV, which is somewhat divergent from the mammalian norm. Clearly SMI33 is a useful probe capable of detecting as few as two neurofilament KSP repeats and is relatively insensitive to the exact organization of these sequences. SMI32, a monoclonal antibody which selectively recognizes the dephosphorylated forms of NF-M and NF-H (Kziezak-Reding et al., 1987; Nukina et al., 1987), recognizes the RH:559-1072 and RH:559-794 clones, but not the RH:846-1072 clone, suggesting that this antibody either requires more KSP sequences or some other feature of the highly-regular KSP repeats found only towards the N-terminus of the NF-H KSP segments. This antibody weakly recognizes RM:549-845, which contains the NF-M KSP2 sequences (Figure 3-5), and does not stain the chicken NF-M clone. This antibody therefore appears to be directed primarily against longer or more organized NF-H KSP sequences, but has a limited ability to bind to NF-M KSP sequences. This data provides the first detailed characterization of N52, and confirms and extends our understanding of the binding requirements of SMI32 and SMI33.

Our results localize the epitopes for RMO1, RMO59 and NN18, a widely used NF-M monoclonal antibody, to the extreme carboxyterminus of NF-M between amino acids 677 and 732. As noted above, we have previously found that certain neurons stain poorly or not at all with NN18 although they stain well with other NF-M antibodies (Weber et al., 1983, Eaker et al., 1990). We can now conclude that some modification of the

region between 677 and 732 is responsible. This could involve unusual protein folding, post-translational modification or the presence of a binding protein, and may be of functional significance. The work described here is a necessary first step towards understanding what this modification might be. It will also be useful in the understanding of some previous surprising results, for example the finding of an NN18 immunoreactive microtubule-associated protein in crayfish neurons (Weaver and Viancour, personal communication) and a strongly NN18 immunoreactive 58kDa protein (NF5) in the myoplasm of developing ascidia (Swalla et al., 1991).

The production of the rabbit polyclonal anti-NF-H KEP and the two KEP monoclonals 3G3 and 5B8 represents a significant addition to the neurofilament antibody repertoire. These NF-H specific antibodies will allow a better determination of NF-H staining in tissue than the KSP reactive antibodies previously available. These unique probes may also prove useful in determining the possible effector role of this previously indistinguishable domain.

Several of the antibodies used here have been shown to recognize epitopes in the tails of NF-M and/or NF-H, but are unable to recognize non-phosphorylated neurofilaments. This family includes SMI31, SMI34, NE14, NF1, NL34, NC43, RT97 and 8D8. Their failure to recognize our fusion proteins is therefore completely in line with these previous findings. Some members of our previous panel of antibodies were either

unaffected or showed only a partially-reduced ability to recognize enzymatically dephosphorylated neurofilaments (Shaw et al., 1984, 1986). NL34 and NM46 belong to this group; although they recognize chymotryptic tails of NF-M and NF-H, they were both completely negative on the fusion proteins tried here. It is possible that these antibodies recognize epitopes containing phosphate groups which are very difficult to remove enzymatically; it is known that enzymatic dephosphorylation does not remove all phosphate groups from neurofilaments (Georges et al., 1986). NA34 is an unusual antibody which recognizes enzymatically dephosphorylated NF-M and NF-H weakly in most species, but stains NF-M strongly in pig (Shaw et al., 1986). NA34 stained RH:559-1072 and RH:559-794, but only very weakly. We do not understand why this antibody fails to stain the NF-M fusion proteins, but can at least conclude that it has some capacity to stain NF-H KSP repeats.

Our polyclonal antibody against the NF-M KE segment stains chicken NF-M strongly and specifically (Figure 3-2). In contrast, the antibodies to the rat KEP and KSP segments stained mammalian NF-H strongly, but recognize chicken NF-H very weakly. We have previously shown that the KEP segment of the NF-H tail is much less conserved in primary sequence than the KE segment of NF-M and proposed that these two regions are distinct enough in sequence to be structurally unrelated (Shaw, 1989). Directly in line with these suggestions we now

show that the KEP segment of NF-H is immunologically much more variable across species boundaries than the KE segment of NF-M, and that the two regions show no immunological cross-reactivity with any of the antibodies employed here. The immunological conservation of the NF-M KE segment documented here suggests structural conservation in line with our proposal that this region subserves an important function (Shaw, 1989). Finally we can speculate that chicken NF-H, for which we currently have no sequence data, will be fairly divergent from the mammalian prototype in the regions homologous to the NF-H KSP and KEP segments.

We noted that the anti-NF-H KSP antibody showed strong staining for cow, but not pig or rat NF-M. This result is in line with an observation we made several years ago; all of our phosphate dependent monoclonal antibodies specific for NF-H also strongly recognize cow NF-M but not NF-M from other mammals (Shaw et al., 1984). We made this odd finding in several different individual cows, and other groups also have noted this phenomenon (Lee et al., 1986). In the meantime we have found that many of the phosphate-dependent antibodies to NF-H recognize phosphorylated KSP sequences (unpublished). We now show that antibody raised against mammalian NF-H KSP repeats strongly stains bovine NF-M. However, polyclonal and monoclonal antibodies to the KE segment all recognize bovine NF-M, so that other regions of the bovine NF-M tail are probably not unusual. Taken together a possible explanation

for these findings is that the KSP repeats of bovine NF-M are much more related to those of the typical NF-H sequences than in other mammals, but that other parts of the bovine NF-M tail do not deviate from the mammalian norm. It will be interesting to read the bovine NF-M sequence should it become available.

The production of fusion proteins like those described here will allow the mapping of other neurofilament antibodies and the production of more antibodies of predefined specificity. The fusion proteins and antibodies raised against them will be valuable probes in future studies of the structure, dynamics and functions of neurofilament tails. The novel polyclonal antisera should also be ideal for screening cDNA expression libraries to clone neurofilament or neurofilament-related proteins, and will also be very useful for immunocytochemical double-labelling and microinjection studies.

CHAPTER 4
IMMUNOHISTOCHEMICAL ANALYSIS OF NF DISTRIBUTION IN RAT NERVOUS SYSTEM

Introduction

Antibodies to neurofilament proteins are often used as markers for neurons. It is generally recognized that not all neurons contain neurofilament triplet proteins. Many studies have looked at neurofilament triplet protein expression in various regions of the rat nervous system, but have come up with different conclusions, especially in the cerebellum (Matus et al., 1979; Shaw et al., 1981; Trojanowski et al., 1985; Shaw et al., 1986; Vitadello and Denis-Donini, 1990). Because neurofilaments, when they are present, tend to be phosphorylated in the axonal compartment but not phosphorylated in the somatodendritic compartment, much of the initial confusion appeared to be resolved by considering whether the antibody's staining is dependent upon the phosphorylation state of the neurofilaments (Goldstein et al., 1983; Sternberger and Sternberger, 1983; Durham, 1990). In addition, the use of subunit-specific antibodies also makes the staining results more intelligible (Shaw et al., 1981; Trojanowski et al., 1985; Carden et al., 1987). Despite these improvements significant differences in results are still

evident and remain unresolved. Our panel of phosphate-independent, subunit- and domain-specific polyclonals and monoclonals should provide data that are more reliable than previously possible.

Methods

For the brain experiments adult cats and Sprague Dawley rats were anaesthetized with pentabarbitol and perfused with heparinized normal saline followed by 4% paraformaldehyde in PBS. Brains were dissected out, post-fixed in the same solution for four to twelve hours at 4°C, transferred to a 20% sucrose solution at 4°C for 6 hours and then placed in a 30% sucrose solution at 4°C for 24 hours. Brains were then quick-frozen in liquid nitrogen-cooled isopentane and embedded for cryostat sectioning. Sections of 6-12 µm were collected either into PBS (for free-floating incubations) or onto gelatin-subbed slides that were then kept frozen at -20°C until staining. Some rat brains were prepared by quick freezing without fixation to check for fixation artifacts. A few Long-Evans rats were used to verify findings in a separate strain.

For examination of structures intimately associated with bone, such as olfactory epithelia and organs of the inner ear, tissues were placed in a 4°C solution of 7.5% EDTA (pH=8.0) for at least 2 days, following perfusion and at least 1 hour post-fixation in 4% paraformaldehyde.

Whole-mounts of retina were prepared by removing the eyes from perfused animals and removing the cornea and lens to expose the retina to the post-fixation solution (4% paraformaldehyde) for an overnight incubation at 4°C. Retinas were usually detached and could be then be easily dissected from the eye for free-floating immunostaining.

For the myenteric plexus experiments segments from the small intestine of neonatal [ages in days: 0(newborn), 4, 7, and 21] and adult (age 60-120 days) rats were taken and prepared in either of two ways. In the first method, samples were frozen in supercooled isopentane, mounted and 5-8 µm frozen sections were cut. Sections were collected on glass slides and fixed with acetone for staining. In the second method, intestine was threaded onto glass capillary tubing and, under the dissecting microscope, the longitudinal muscle layer and accompanying myenteric plexus were removed. The longitudinal muscle/myenteric plexus layer was pinned on Sylgard (Brownell Electric, Orlando, Fl) and fixed overnight in Zamboni's solution (4°C) (Costa et al., 1980; Stefanini et al., 1967). The next day, the tissue was washed free of the Zamboni's fixative using 80% ethanol and then the tissues were dehydrated and rehydrated through graded alcohols prior to staining as the whole mount preparations. Additionally, rat brain from the different age groups was taken and prepared using the two fixation methods described above and sections were immunostained as controls.

Immunohistochemistry was performed either on free-floating tissue in solution or on sections on slides that had been thawed and encircled with a PAP pen (The Binding Site Inc., San Diego, CA). Tissues were blocked with 0.1% BSA and 0.3% Goat serum in PBS for 20 minutes at 37°C. Following a PBS rinse, primary antisera, often containing approximately 0.1% Triton X-100, were applied for at least 45 minutes at 37°C (or overnight at 4°C) and then washed three times in PBS for 10 minutes at room temperature. Fluorescent secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were applied at final dilutions of 1:40 in 0.1% BSA and 0.3% Goat serum in PBS plus 0.1% Triton for at least 45 minutes at 37°C and washed in PBS three times for 10 minutes at room temperature. Double-label applications were performed in the same manner, but with primary and secondary antibody mixtures. Sections and whole mounts were coverslipped using an anti-bleaching mounting medium (1 mg/ml para-phenylene-diamine in 90% glycerol, 10% Tris 200mM, pH = 7.9), sealed with nail polish and stored at 4°C. Some sections were processed using an avidin-biotin peroxidase enhancement method (Vectastain Elite - Vector Laboratories) of detecting the primaries, rather than using fluorescence.

All polyclonal rabbit sera against the native neurofilaments (H297, H298, H301), the fusion proteins (anti-rat NF-H KSP, anti-rat NF-H KEP, anti-rat NF-M KE, anti-chicken NF-M E/KSP) and some monoclonal ascites fluids (DA2,

NAP4, NN18, SMI32) were used at a final dilution of 1:200 in PBS, whereas hybridoma culture media supernatants for the fusion protein monoclonals were typically used undiluted. The α-internexin monoclonal (#135) was a gift from Dr. Ron Liem. The GABA antibody (Eugenetech, Inc.) was used at 1:750 for a primary incubation of at least 3 days at 4°C.

Acetycholinesterase (AChE) staining was performed on free-floating sections followed by the immunohisto-fluorescent labelling exactly as described above. AChE staining involved pre-incubation in 0.5 M Na citrate, 0.3 M $CuSO_4$, 0.05 M $K_3Fe(CN)_6$, 0.1% Triton X-100 in 0.1 M Acetate buffer (pH=6.0) for 20 minutes at room temperature, followed by a 15 minute incubation in 20 ml of pre-incubation solution containing 1.4 mg ethopropazine and 14 mg acetylthiocholine iodide, a 5 minute wash in 0.05 M TRIS (pH=7.6) and developing in 0.5% DAB in PBS plus 0.1% Triton (Geneser-Jensen and Blackstad, 1973).

Alkaline phosphatase treatments of unfixed and fixed sections were incubated in E. coli Alkaline phosphatase (Sigma #P-5931) at 2 mg/ml in TBS for 4 hours at 37°C, overnight (approximately 12 hours) at 4°C and then for an additional hour at 37°C prior to immunostaining.

Results

Overview. Neurofilament triplet staining was evaluated in many cell types and fiber tracts; most structures were similarly immunoreactive for NF-L, NF-M and NF-H (Table 4-1).

TABLE 4-1. STRUCTURES SHOWING NEUROFILAMENT IMMUNOREACTIVITY

Cortex - Pyramidal cells

Hippocampus
Dentate granule- NF-M >>> NF-H and NF-L
Dentate Interneurons- NF-H and NF-L > NF-M
CA Pyramidals- NF-H and NF-L > NF-M
CA Interneurons- NF-H and NF-L > NF-M
Mossy fibers- NF-M >> NF-H or NF-L

Cerebellum
Purkinje cells- Anti-NF-H KSP >>> all other NF antibodies
Granule cells- Anti-NF-H KSP positive in cat, but not rat
Parallel fibers[1]- Lower third are positive for NF triplet; NF-H > NF-L > NF-M
Basket cells- Anti-NF-H KSP positive in cat, but not rat
Basket cell axons- Anti-NF-H KSP negative in cat
Stellate cells- Anti-NF-H KSP positive in cat, but not rat
Large Golgi cells- Anti-NF-H KSP positive in cat, but not rat
Small Golgi cells[2]- NF-H > NF-L >>> NF-M; seen only in rat, not cat
Lugaro cells- Anti-NF-H KSP positive in cat and rat
Deep cerebellar nuclei
Mossy and Climbing fibers

Striatum - Internal capsule

Tectum - Posterior and Superior colliculi

Tegmentum - Locus coeruleus and Substantia nigra

Trigeminal nuclei - (mesencephalic, motor, principal, spinal)

Pontine nuclei

Spinal Cord - tracts and α-motor neurons

Dorsal Root Ganglia

Retina - Ganglion cells and Horizontal cells

Spiral Ganglia
Type I neurons- Immunonegative with anti-NF-H KEP, 3C3 and 5B8
Type II neurons

Vestibular Ganglia and Organs - Hair cells and Afferent endings (Saccule, Utricle, Ampules)

Olfactory Bulb
Glomeruli, Mitral cells, Tufted cells- Anti-NF-H KSP > all other NF antibodies

Olfactory epithelial neurons[3]

All of the structures listed above were positive for NF-L, NF-M and NF-H, except where noted otherwise.

[1]Although all parallel fibers were immunopositive for α-internexin, only the larger diameter fibers in the deep third of the molecular layer were NF triplet positive.
[2]With all NF-M monoclonals small Golgi cells appeared immunonegative; only a very weak signal was detected with NF-M polyclonals.
[3]Only a minority of olfactory epithelial neurons from P4 rat were NF immunopositive.

In some cases, however, there are notable differences in the staining patterns for the different subunits. As is consistent with the existing literature, the overall pattern most often seen was that of a dephosphorylated NF-H KSP antibody staining preference in cell bodies and a fiber tract selection by phosphorylated NF-H KSP antibodies (Figure 4-1). The NF-H KEP, NF-M KE, and NF-L antibodies were particularly striking, however, in that even though there was a significant cell body reaction, many of them stained fibers more intensely than cell bodies (Figures 4-1 to 4-5 and 4-12). Unlike antibodies directed against the phosphorylated KSP regions of NF-M and NF-H, the staining pattern of NF-H KEP, NF-M KE and NF-L antibodies are unaffected by alkaline phosphatase treatments; perhaps this preferential staining of axons by markers for non-phosphorylated epitopes reflects a quantitative difference in the concentration of neurofilament proteins in the axonal processes compared to the somatodendritic compartment.

Cerebellum. Beyond the general patterns of somatodendritic versus axonal staining, two new and unusual findings were discovered in the rat cerebellum. Firstly, in horizontal sections we were able to see a subpopulation of parallel fibers (the larger caliber ones located deeper in the molecular layer) that were immunoreactive to NF-H and NF-L (Figure 4-2), and to a lesser degree NF-M (Figure 4-3); this NF triplet positive subpopulation is compared by double-label

to the entire veil of parallel fibers, not just large caliber ones, stained by an α-internexin antibody (Figure 4-4). Secondly, and of much more interest, we found staining of what appear to be a population of previously undescribed cells that we think correspond to what have been called small Golgi neurons at the electron microscopic level (Palay and Chan-Palay, 1974). These neurons were even more intriguing because they demonstrated a difference in the amounts of NF-L, NF-M and NF-H immunoreactivity, with NF-H consistently being much stronger. In fact α-internexin (Figure 4-5) and most NF-M (Figure 4-6) antibodies appeared negative, but NF-L and all NF-H antibodies, irregardless of the phosphate-dependence or prior phosphatase treatment, robustly stained these cell bodies (Figures 4-5 through 4-8). This finding is similar, but not identical, in Purkinje cells, in which the soma and dendritic trees also stain strongly for NF-H, but not for NF-M, NF-L and α-internexin; this difference is only seen, however, by using antibodies against the dephosphorylated NF-H KSP (Figures 4-1, 4-4 to 4-6, 4-10 and 4-11). Because the small Golgi neurons were not previously described as being strongly immunoreactive for NF-H, considerable effort was expended to confirm their identity. The cells were of the right size and distribution (granular layer, most concentrated in vestibular lobules IX and X) (Figures 4-7 and 4-8). In addition, AChE staining distinguished a subset of nodular mossy fibers (as described by Brodal and Drablos in 1963)

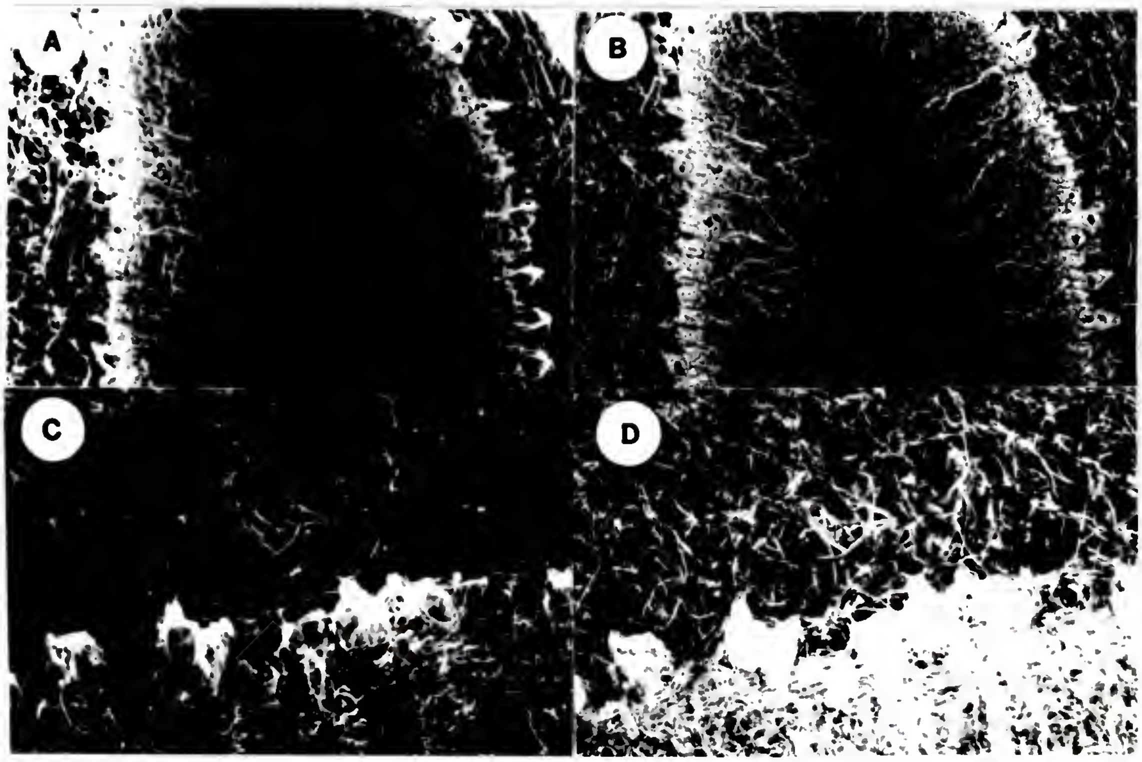

Figure 4-1. Axonal versus somatodendritic staining patterns of NF antibodies. A and B: Double-label immunofluorescence of a sagittal section of rat cerebellum using a monoclonal to the NF-H KEP, 3G3 (A) and the anti-NF-H KSP (B). C and D: Double-label of a horizontal section of rat cerebellum stained with a monoclonal against phosphorylated NF-H KSP, NAP4 (C) and the anti-NF-H KSP serum (D), which prefers dephosphorylated NF-H. Approximate magnification: A and B = 40x; C and D = 100x.

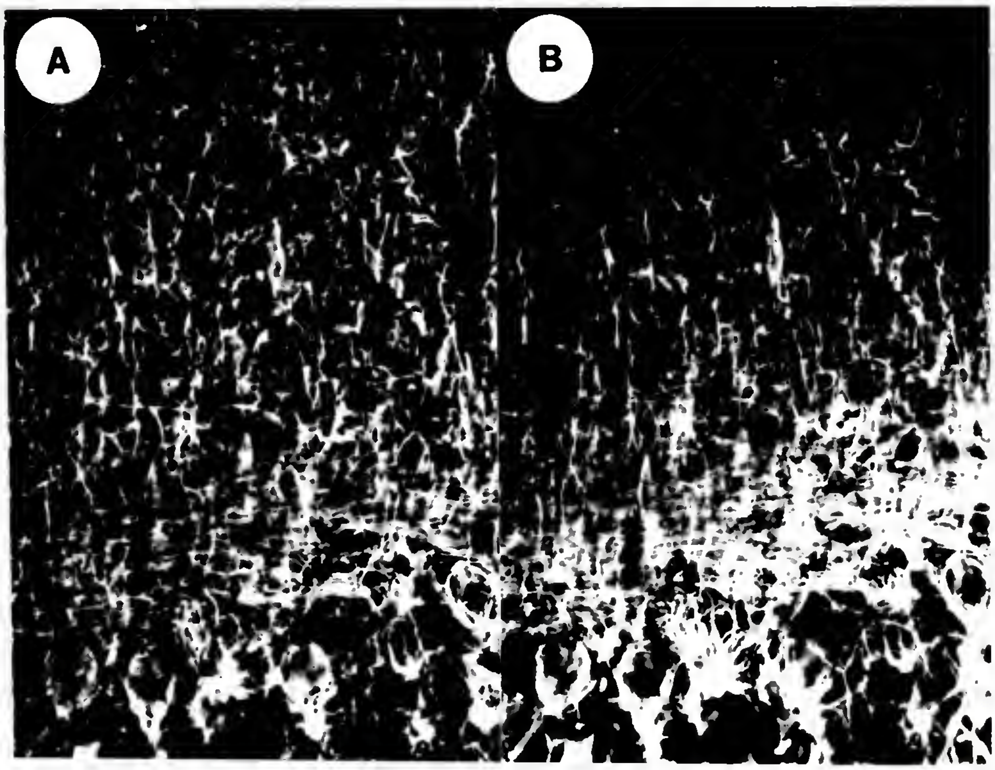

Figure 4-2. NF-H and NH-L antibody staining of a subpopulation of parallel fibers. Double-label immunofluorescence of a horizontal section of rat cerebellum using the polyclonal anti NF-H KEP (A) and the monoclonal to NF-L, DA2 (B). Approximate magnification: 100x.

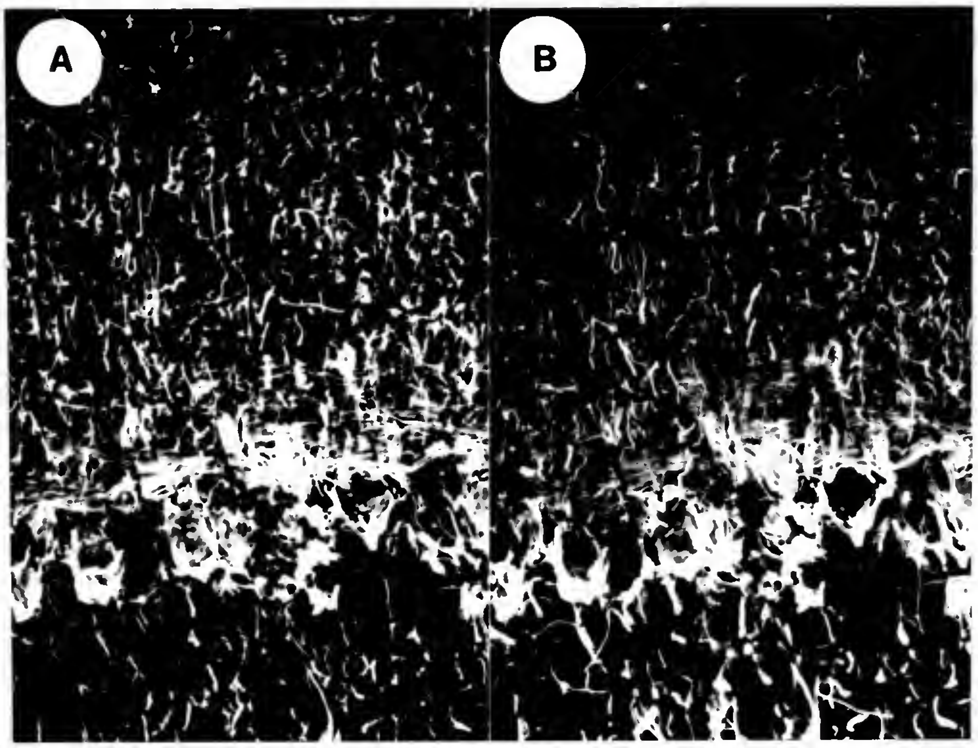

Figure 4-3. NF-H and NF-M antibody staining of a subpopulation of parallel fibers. Double-label immunofluorescence of a horizontal section of rat cerebellum using the polyclonal anti NF-H KEP (A) and the monoclonal to NF-M, 3H11 (B). Approximate magnification: 100x.

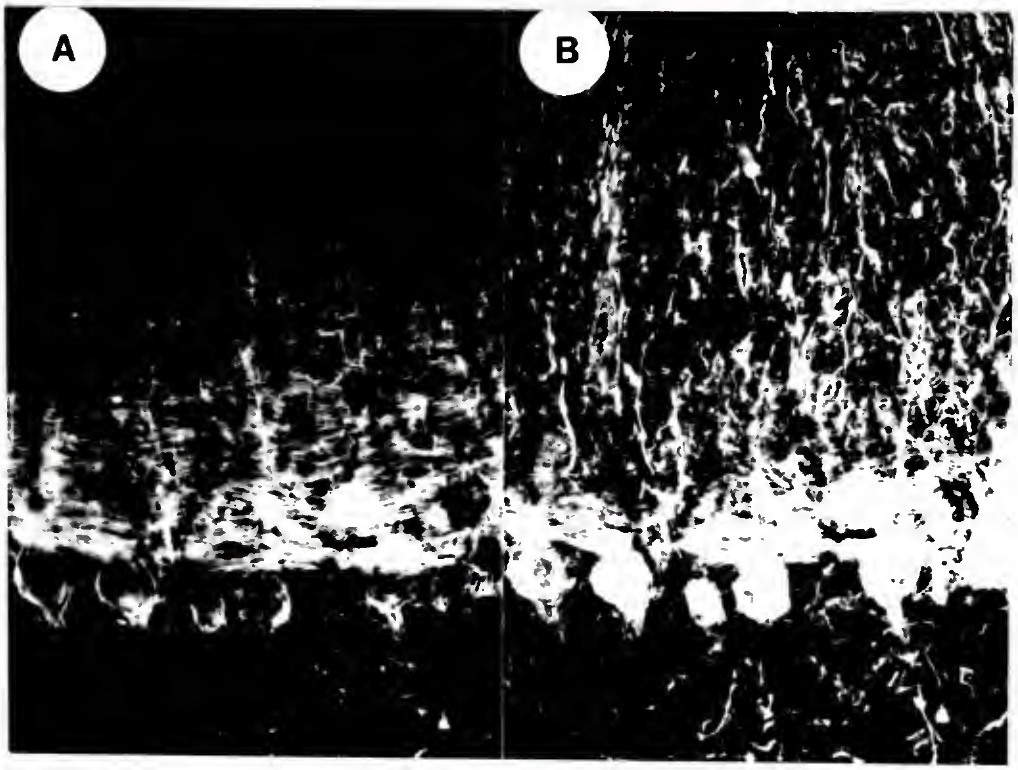

Figure 4-4. NF-H and α-internexin antibody staining of parallel fibers. Double-label immunofluorescence of a horizontal section of rat cerebellum using the polyclonal anti-NF-H KSP (A) and the monoclonal against α-internexin, #135 (B). Compared to the #135 monoclonal which stains all off the parallel fibers, the NF-H antiserum only reveals a subpopulation of parallel fibers in the deep third of the molecular layer. Approximate magnification: 100x.

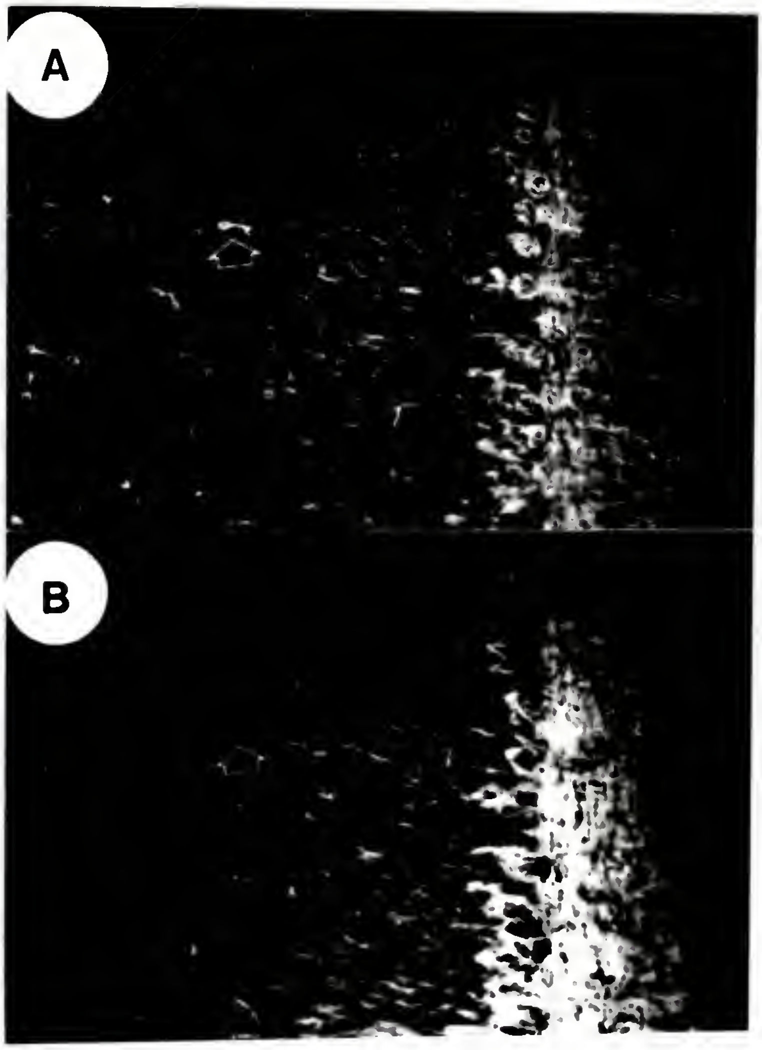

Figure 4-5. NF-H versus α-internexin antibody staining of a horizontal section through the rat nodulus. Double-label immunofluorescence using the α-internexin monoclonal #135 (A) and the polyclonal anti-NF-H KSP (B); The small Golgi neurons are visible only with the NF-H antibody. Approximate magnification: 63x.

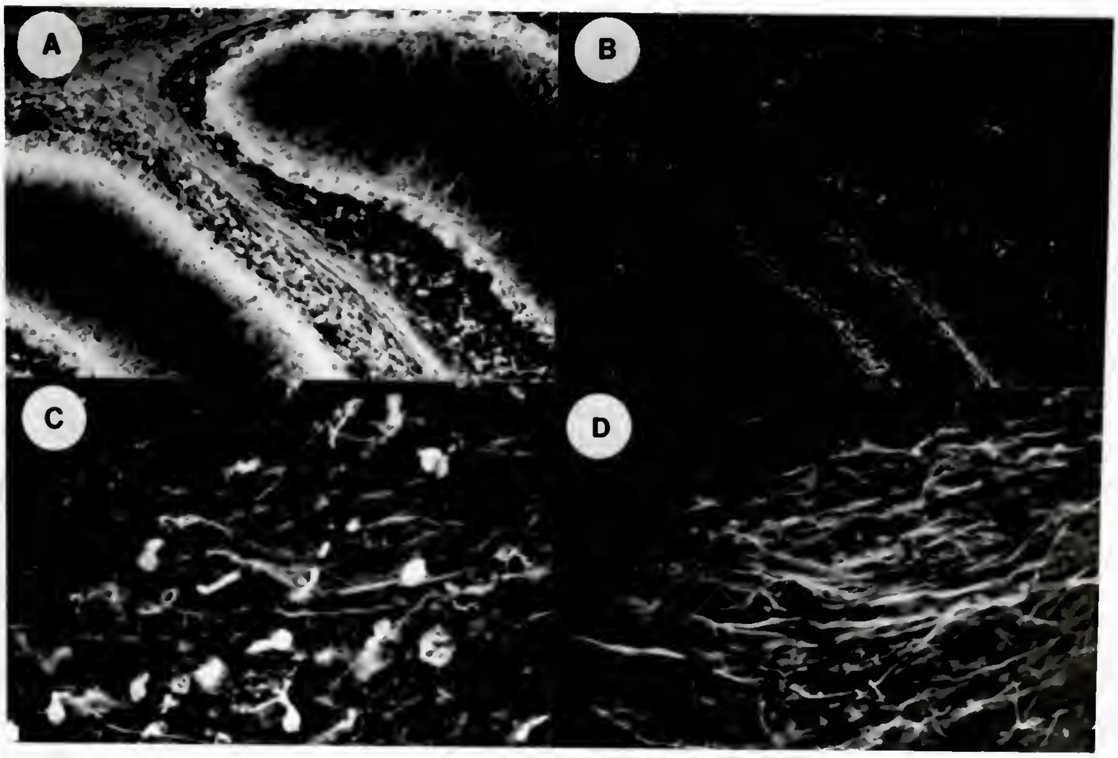

Figure 4-6. NF-H versus NF-M antibody staining of the rat uvula. A and B: Double-label immunofluorescence of a sagittal section with the polyclonal anti-NF-H KSP (A) and NF-M monoclonal NN18 (B). C and D: Double-label immunofluorescence of a sagittal section with the polyclonal anti-NF-H KSP (C) and NF-M monoclonal 3H11 (D). The small Golgi neurons are visible only with the NF-H antibody. Approximate magnification: A and B = 20x; C and D = 100x.

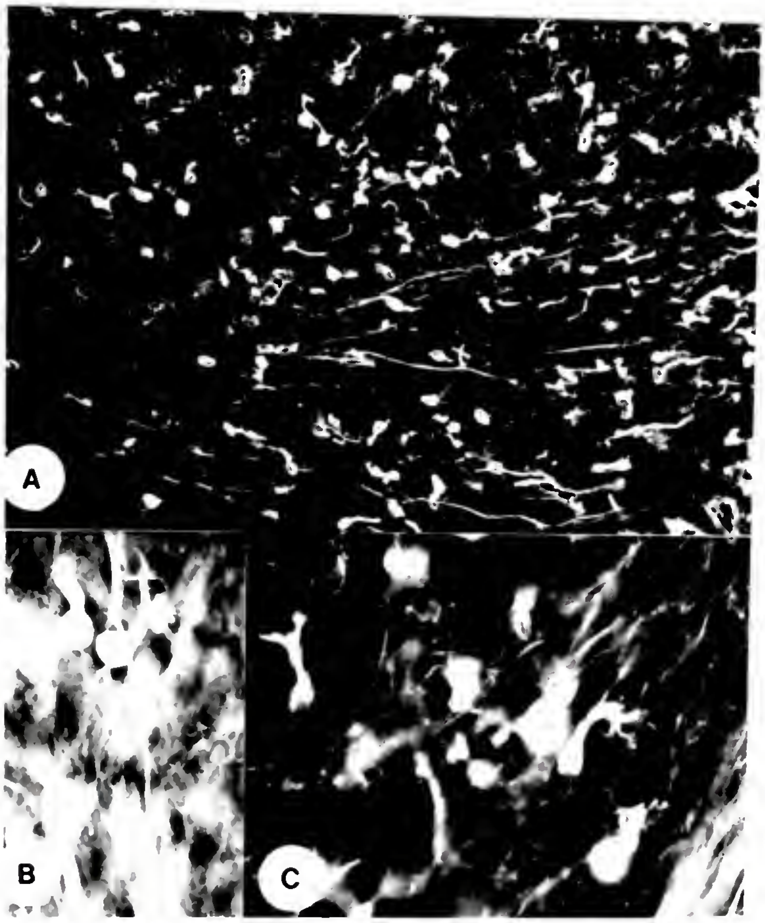

Figure 4-7. NF-H positive small Golgi neurons. Immunofluorescence of the rat nodulus containing small Golgi neurons stained with the anti-NF-H KSP polyclonal. Note the number and morphology of these cells. Approximate magnification: A = 63x; B and C = 200x.

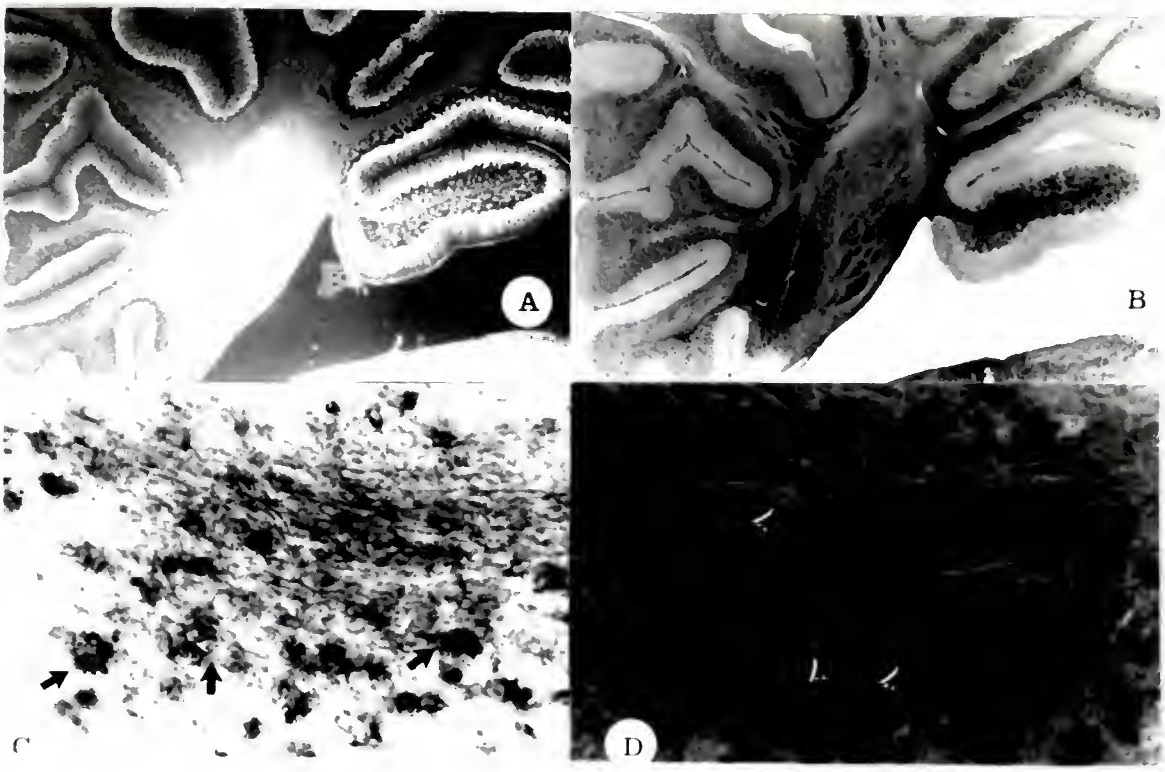

Figure 4-8. Distribution of NF-H positive small Golgi neurons compared to acetylcholinesterase (AChE) positive nodular-type mossy fiber terminals by double-label. A: Immunofluorescence of an anti-NF-H KSP stained sagittal section of rat cerebellum demonstrating that the small Golgi neurons are predominantly found in the granule cell layer of the vestibulocerebellum (lobules IX and X, arrow). B: AChE staining of the same section as in A, showing an enhanced reactivity in the granule cell layer of lobule IX and X as compared to the other lobules. C: AChE positive mossy fiber endings (arrows). D: Double-label of same section as in C, showing the location of NF-H KSP positive small Golgi cells (arrowheads) as well as some antibody-labelled climbing fibers. Approximate magnification: A and B = 20x; C and D = 200x.

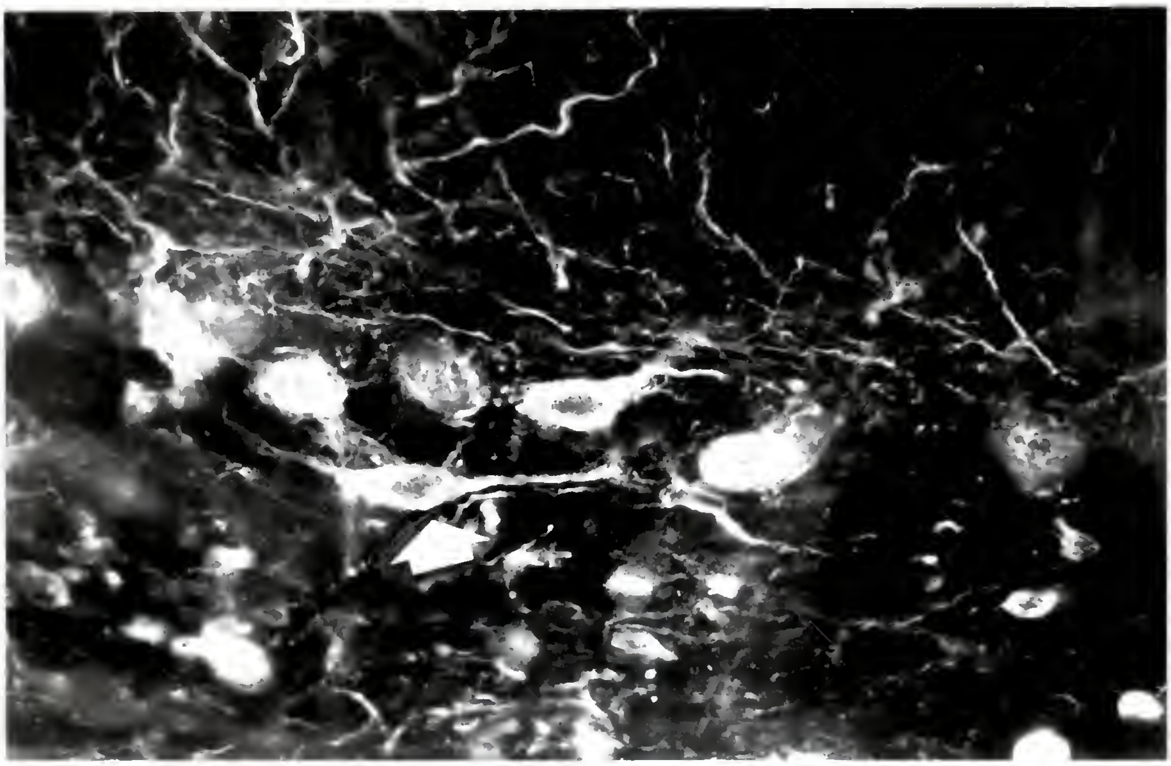

Figure 4-9. Lugaro cell in the rat cerebellum. Immunofluorescent labelling of a Lugaro cell (arrow), as well as some Purkinje cells, some transverse basket cell axons and some small Golgi neurons with anti-NF-H KSP. Approximate magnification: 200x.

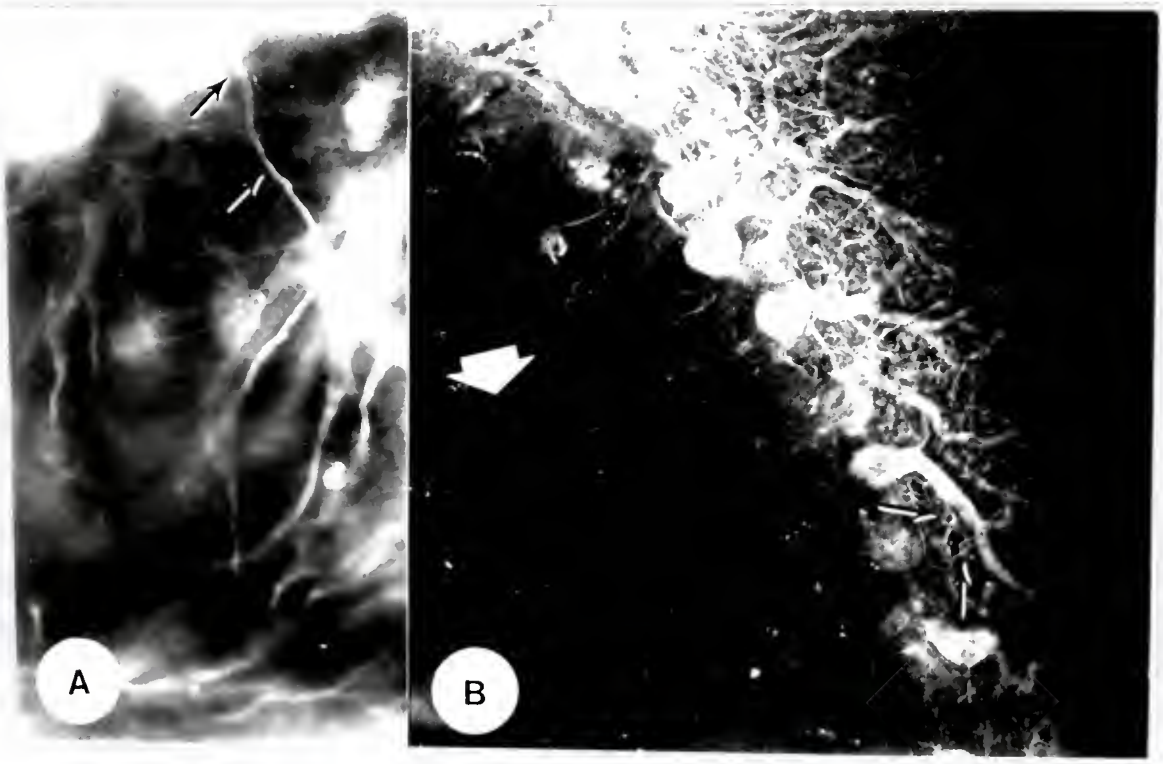

Figure 4-10. Anti-NF-H KSP labelling of cat Purkinje cell axons and a large Golgi neuron. A: Intense staining of an axon (arrows) as it exits a Purkinje cell body. B: Faint staining of a large Golgi neuron (arrow). Approximate magnification: A = 250x; B = 150x.

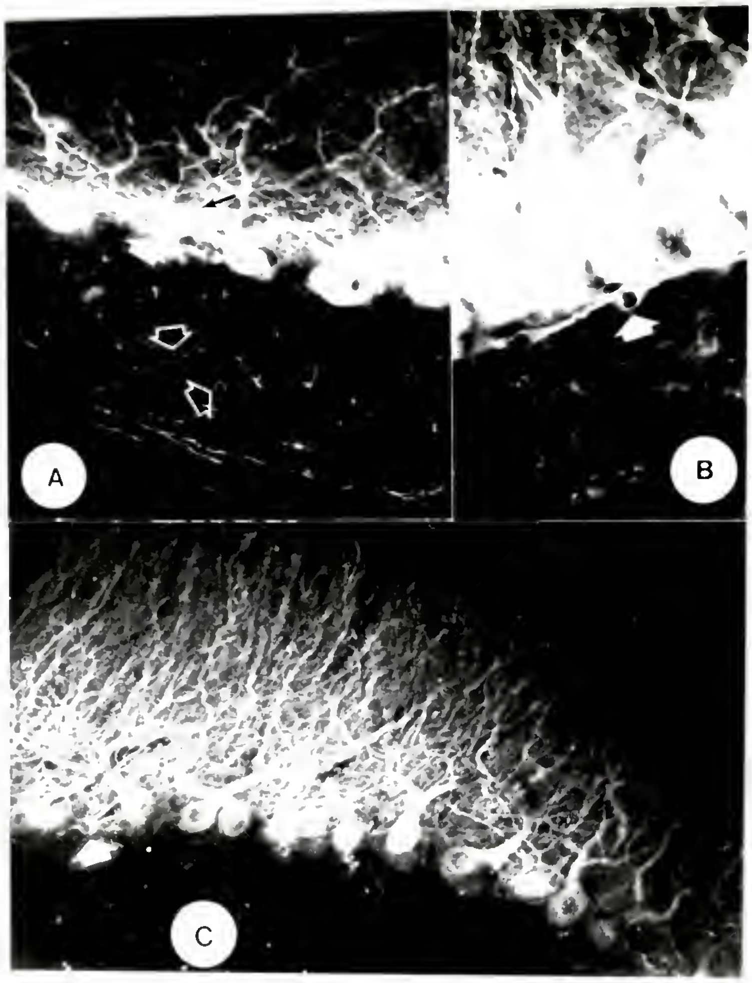

Figure 4-11. Anti-NF-H KSP labelling of cat granule and Lugaro basket cells. A: Granule cells (open arrows) are faintly labelled by neurofilament antibody; note that their processes are thinner than those of small Golgi cells (Figure 4-7) which taper more slowly into a dendritic trunk. B: Staining of an horizontally-oriented interneuron (arrow) at the base of the Purkinje cell layer which is either a Lugaro cell or subclass of basket cell. C: Labelling of two typical basket cell (arrows). Approximate magnification: A = 75x; B = 100x; C = 63x.

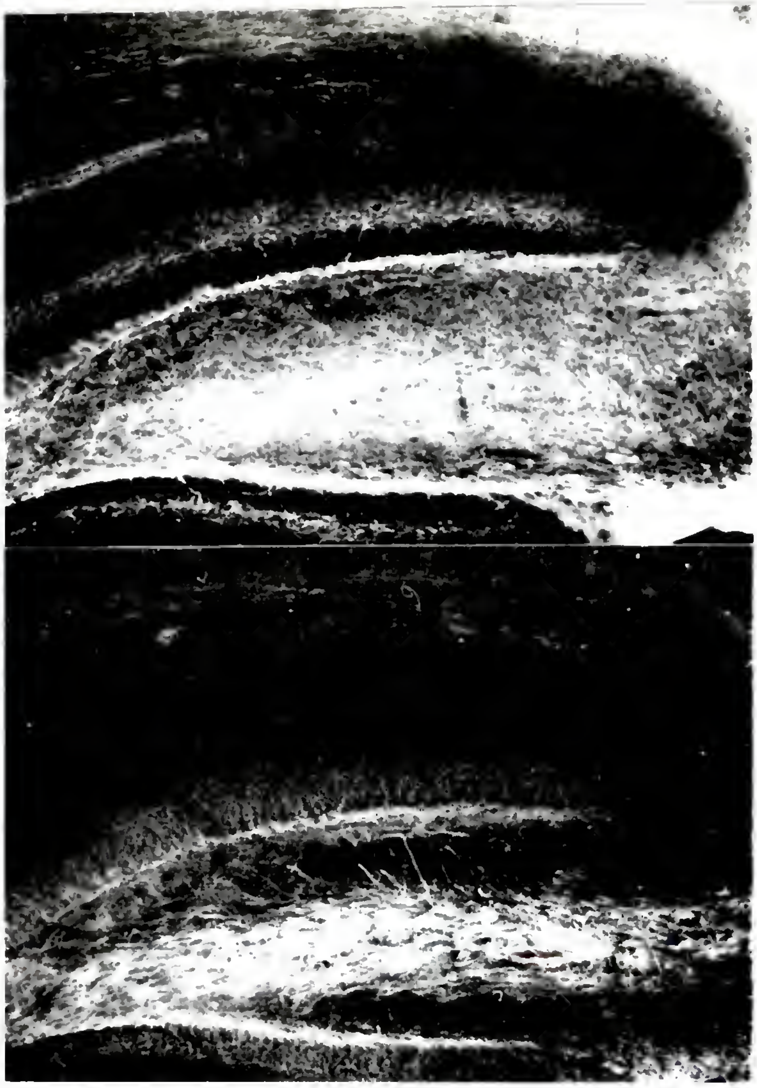

Figure 4-12. Double-label of NF-H versus NF-M immunoreactive patterns in rat hippocampus. Anti-NF-H KSP (A) stains many different hippocampal cell and fiber types, but is less reactive with the dentate granule cell layer and the mossy fibers concentrated in the very center of the dentate. The NF-M antibody 3H11 (B) stains the same section in a nearly complementary fashion, reacting most strongly with the mossy fibers and the dentate granule cell layer. Approximate magnification: 40x.

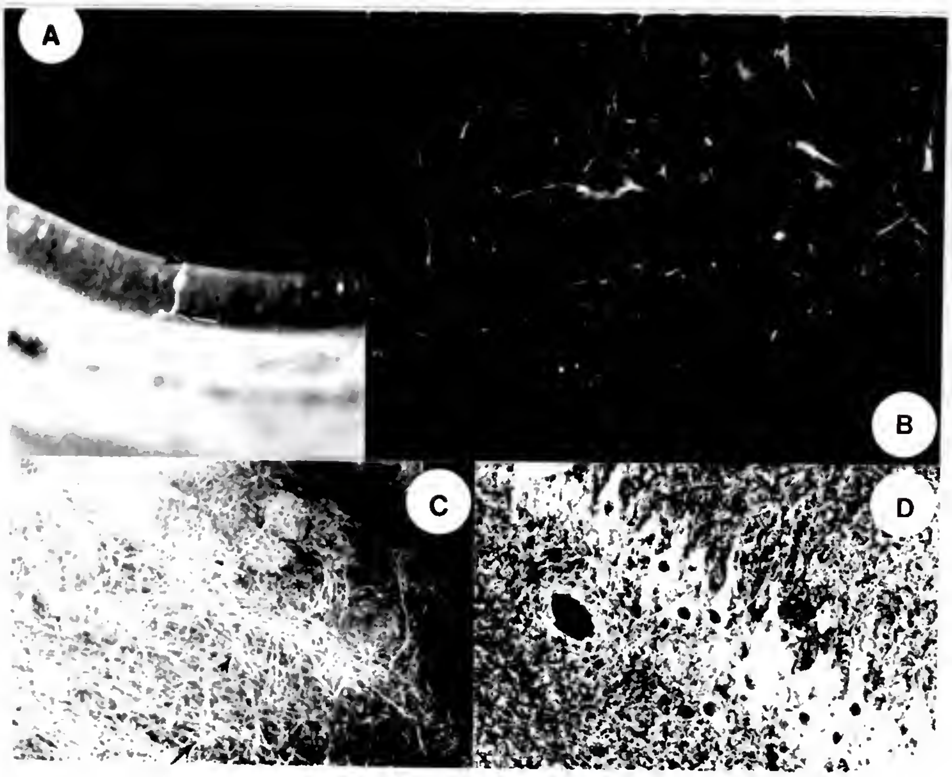

Figure 4-13. Labelling of assorted neurons by NF antibodies. A: Intense labelling of an occasional primary neuron of the P4 rat olfactory epithelium with DA2, a NF-L monoclonal; besides the soma, both the dendritic knob (arrowhead) and the axon (arrow) is visualized by the antibody. B: Staining of a layer V pyramidal neuron from rat cerebral cortex with NF-M KE monoclonal 3H11. C: Intense staining of rat olfactory bulb structures with anti-NF-H KSP polyclonal. Labelled structures include glomeruli (asterisks), mitral cells (arrow) and tufted cells (arrowhead). D: Intense labelling of CA1 pyramidal neurons from rat hippocampus with anti-NF-H KSP serum. Approximate magnifications: A and B = 200x; C = 40x; D = 100x.

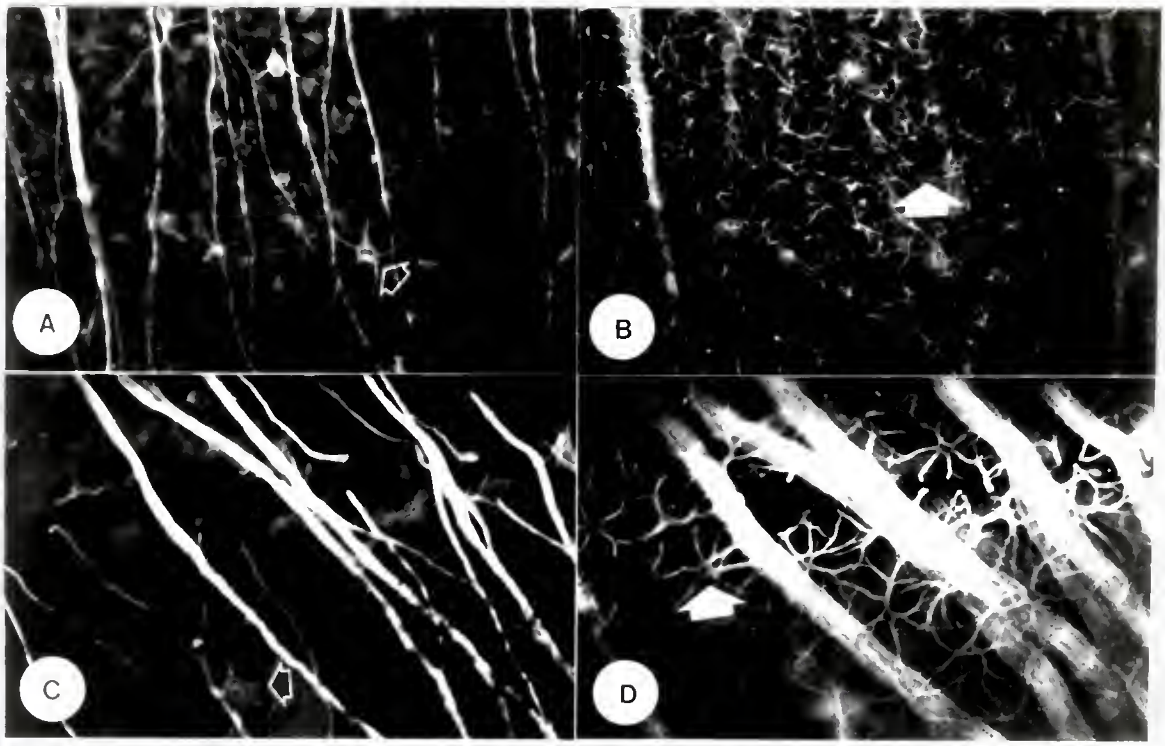

Figure 4-14. Anti-NF-H KSP staining of retinal ganglion and horizontal cells from rat and cat. Visualization of the same area of a whole mount rat (A and B) or cat (C and D) retina in two different focal planes shows staining of ganglion cells (open arrows, A and C) and horizontal neurons (arrows, B and D). Approximate magnification: A and B = 100x; C and D = 200x.

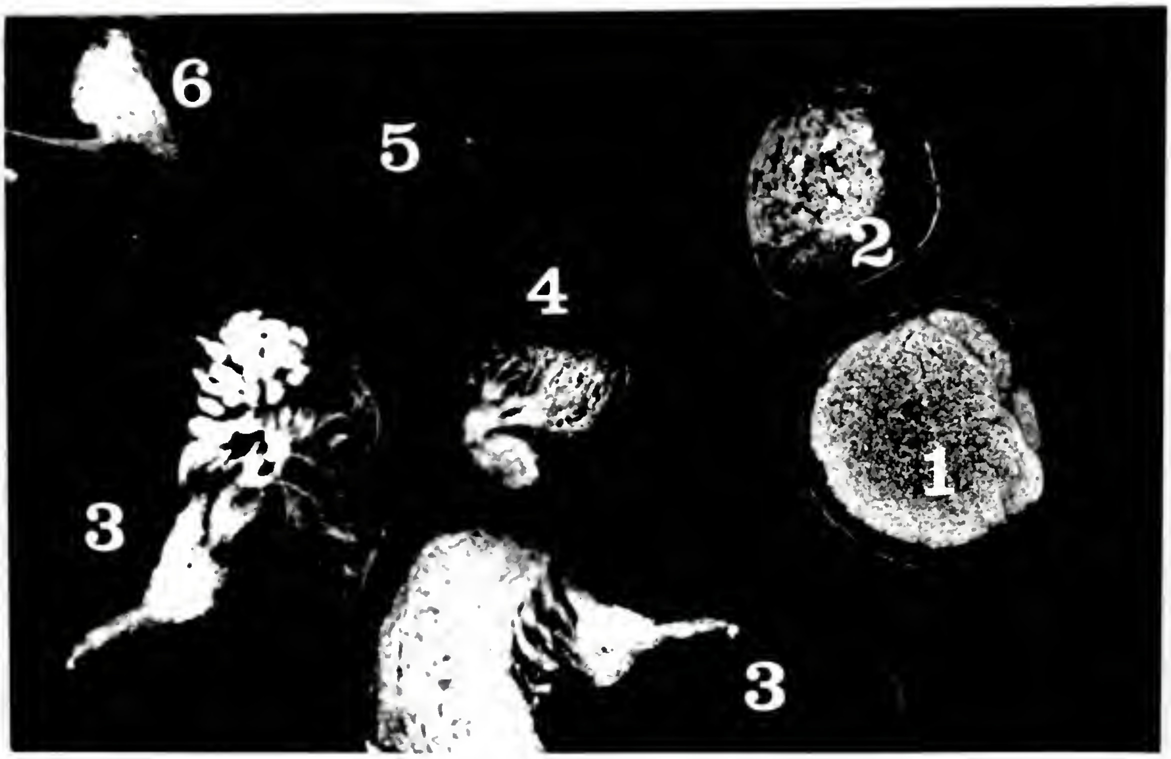

Figure 4-15. Immunofluorescent staining of rat inner ear structures by Anti-NF-H KSP. Structures labelled include: 1) eighth cranial nerve; 2) vestibular ganglion; 3) cochlea; 4) sacculus; 5) utricle; 6) ampulla of semicircular canal. Approximate magnification: 20x.

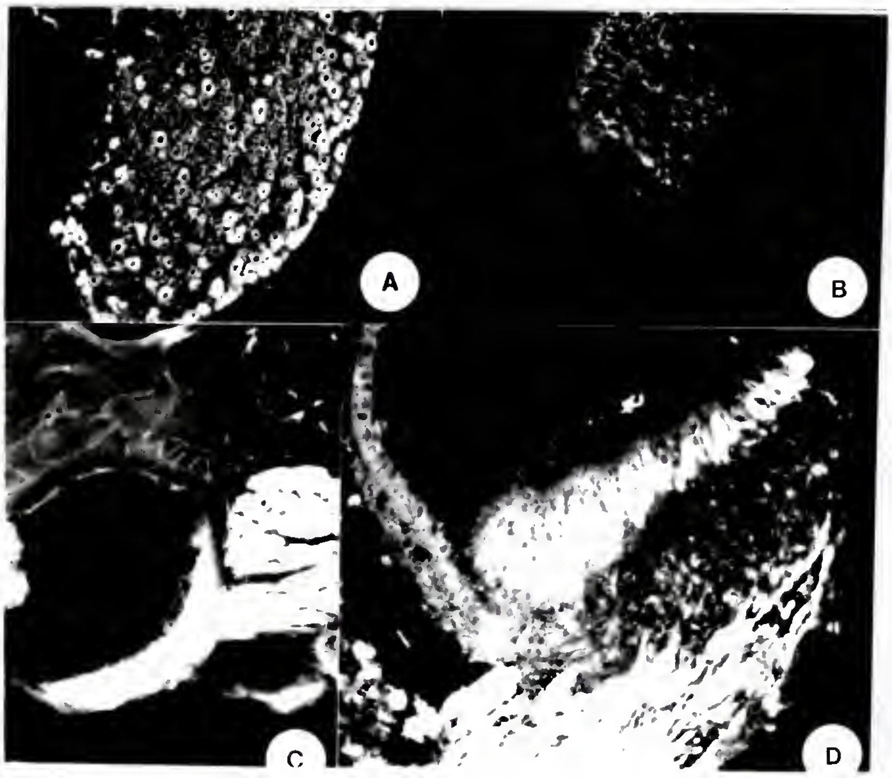

Figure 4-16. Rat vestibular ganglion and organs labelled by NF antibodies. A: Vestibular ganglion stained with anti-NF-M KE. B: NF-M monoclonal NN18 stained calyceal afferent nerve endings for hair cells of the crista of an ampulla. C: NN18 staining of the afferent fibers and calyces around hair cells of the utricular macula. D: Apparent staining of hair cells as well as the afferent fibers of the sacculus by 5B8, a monoclonal against the KEP domain of NF-H. Approximate magnification: A, B and C = 63x; D = 100x.

Figure 4-17. Double-label immunofluorescence of spiral ganglion by Anti-NF-H KEP and NF-M monoclonal NN18. A demonstration of intense labelling of all spiral ganglion neurons and afferent fibers by NN18 (A), but only strong label of fibers by anti-NF-H KEP (B). Approximate magnification: 100x.

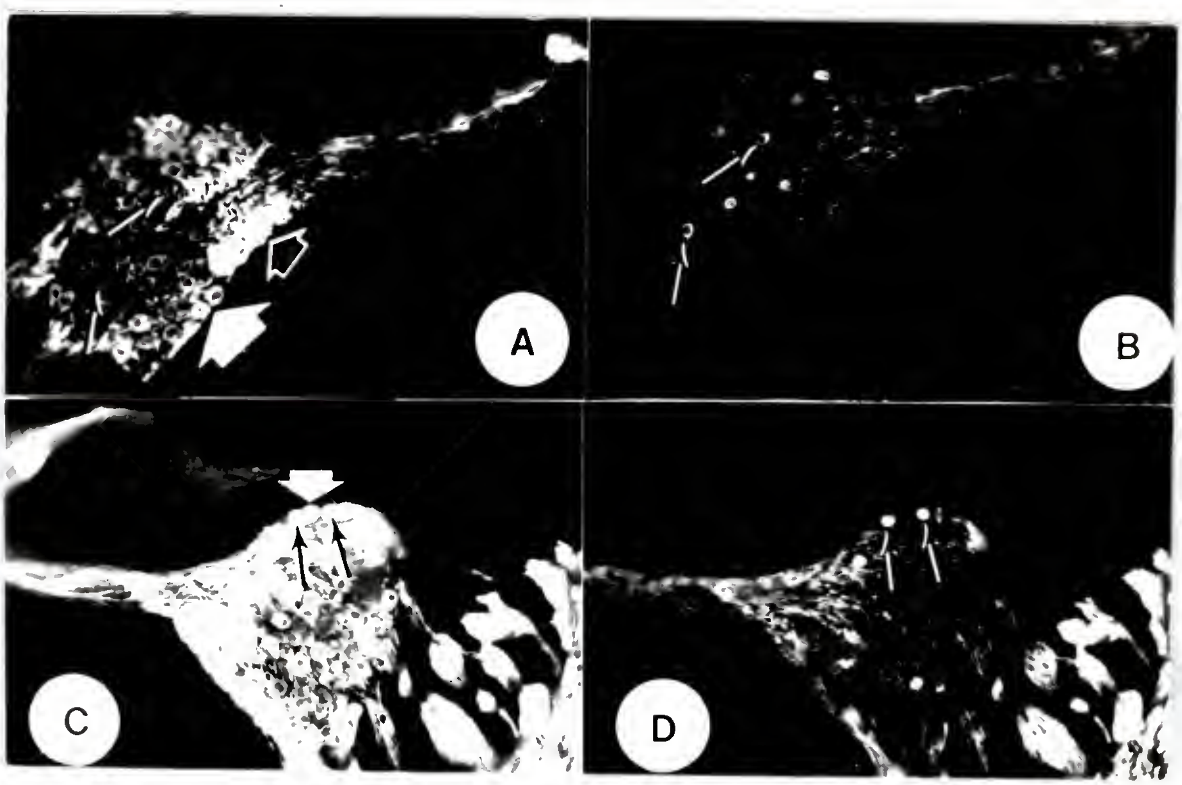

Figure 4-18. Double-label immunofluorescence of rat spiral ganglion by anti-NF-M KE and NF-H KEP monoclonal 5B8. A and B are double-labels of the same section, as are C and D. Although both type I (heavy white arrows) and type II (thin black arrows) neurons are labelled by anti-NF-M KE (A and C), only type II neurons can be seen with 5B8 (B and D). Note that there appears to be a set of fibers which are labelled by the anti-NF-M KE, but not 5B8 (open arrow, A). Approximate magnification: 200x.

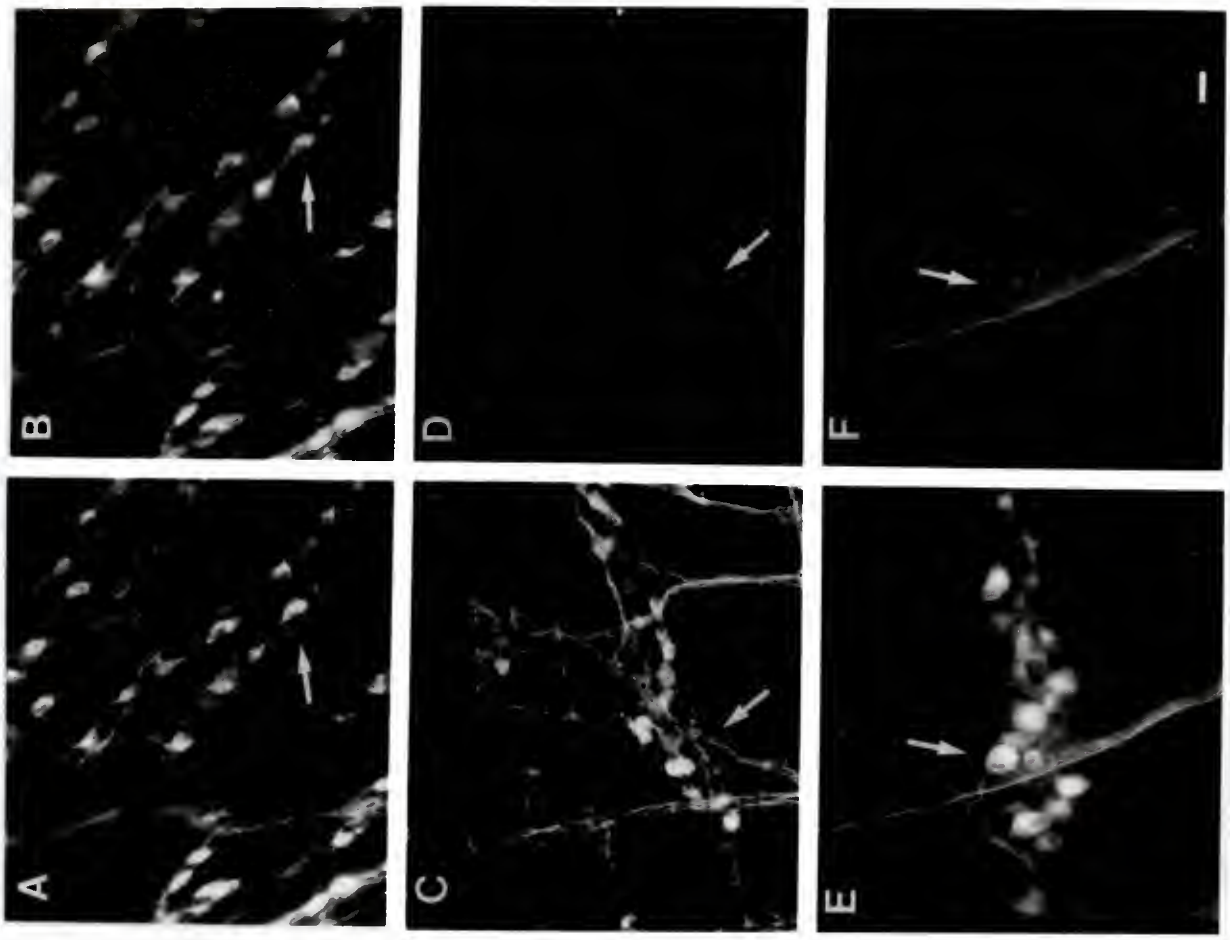

Figure 4-19. Double-label immunofluorescence of myenteric neurons stained with Anti-NF-M KE and NN18. Whole mounts of myenteric plexi stained with anti-NF-M KE (A, C, and E) and NN18 (B, D, and F). Anti-NF-M KE stains myenteric neurons in all ages. Neonate intestine (A and B) shows comparable positive immunostaining in neurons (arrows) with anti-NF-M KE and NN18. Intestine from 7 day old rats (C and D) shows a decrease (to background) of NN18 staining in neurons (arrows). Adult intestine (E and F) demonstrates no detectable NN18 signal in myenteric neurons. Scale bar = 20 μm.

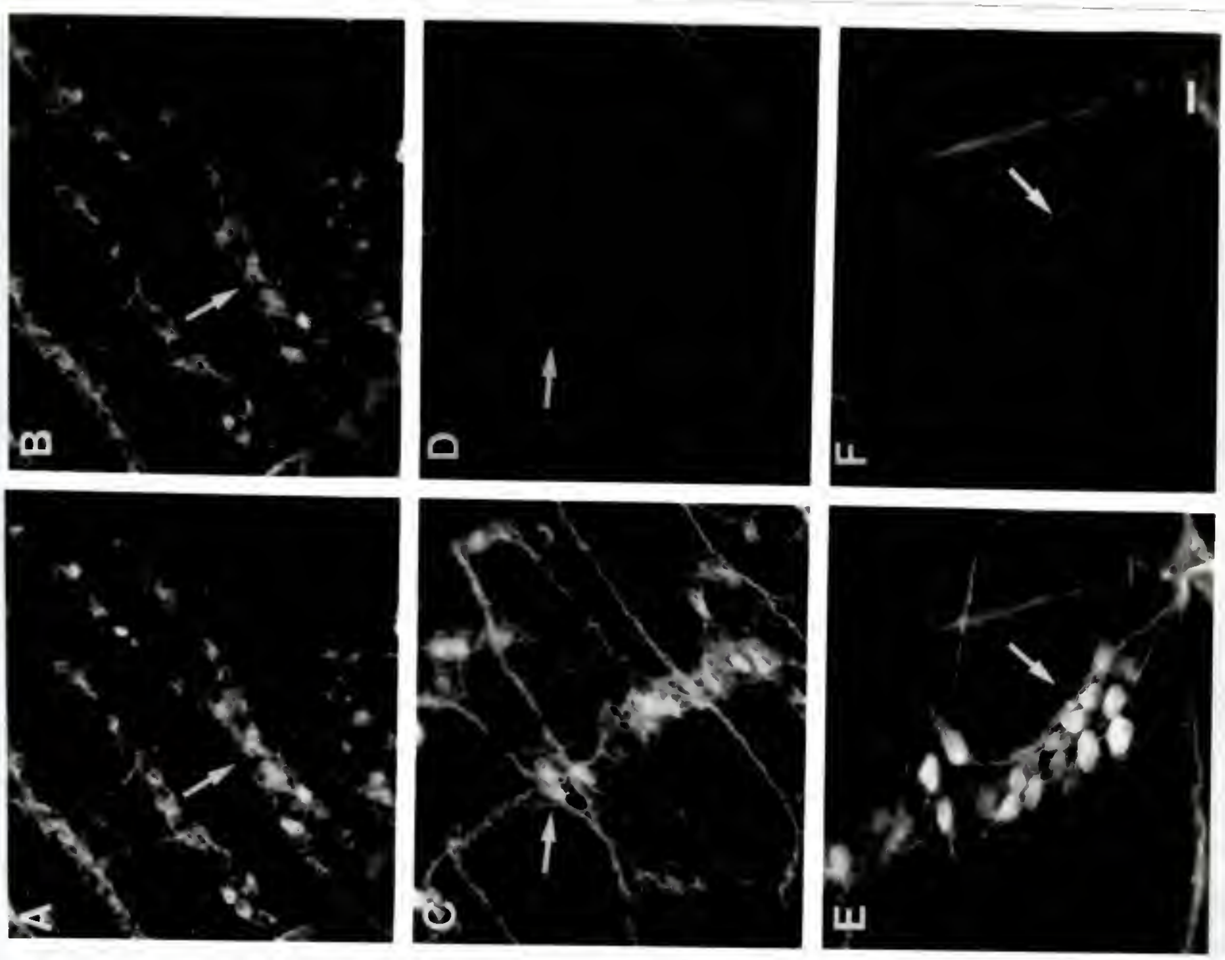

Figure 4-20. Double-label immunofluorescence of myenteric neurons stained with Anti-NF-M KE and RMO1. Whole mounts of myenteric plexi stained with anti-NF-M KE (A, C, and E) and RMO1 (B, D, and F). Anti-NF-M KE stains myenteric neurons in all ages. Neonate intestine (A and B) shows comparable positive immunostaining in neurons (arrows) with anti-NF-M KE and RMO1. Intestine from 7 day old rats (C and D) and adult (E and F) demonstrates no detectable RMO1 signal in myenteric neurons (arrows). Scale bar = 20 µm.

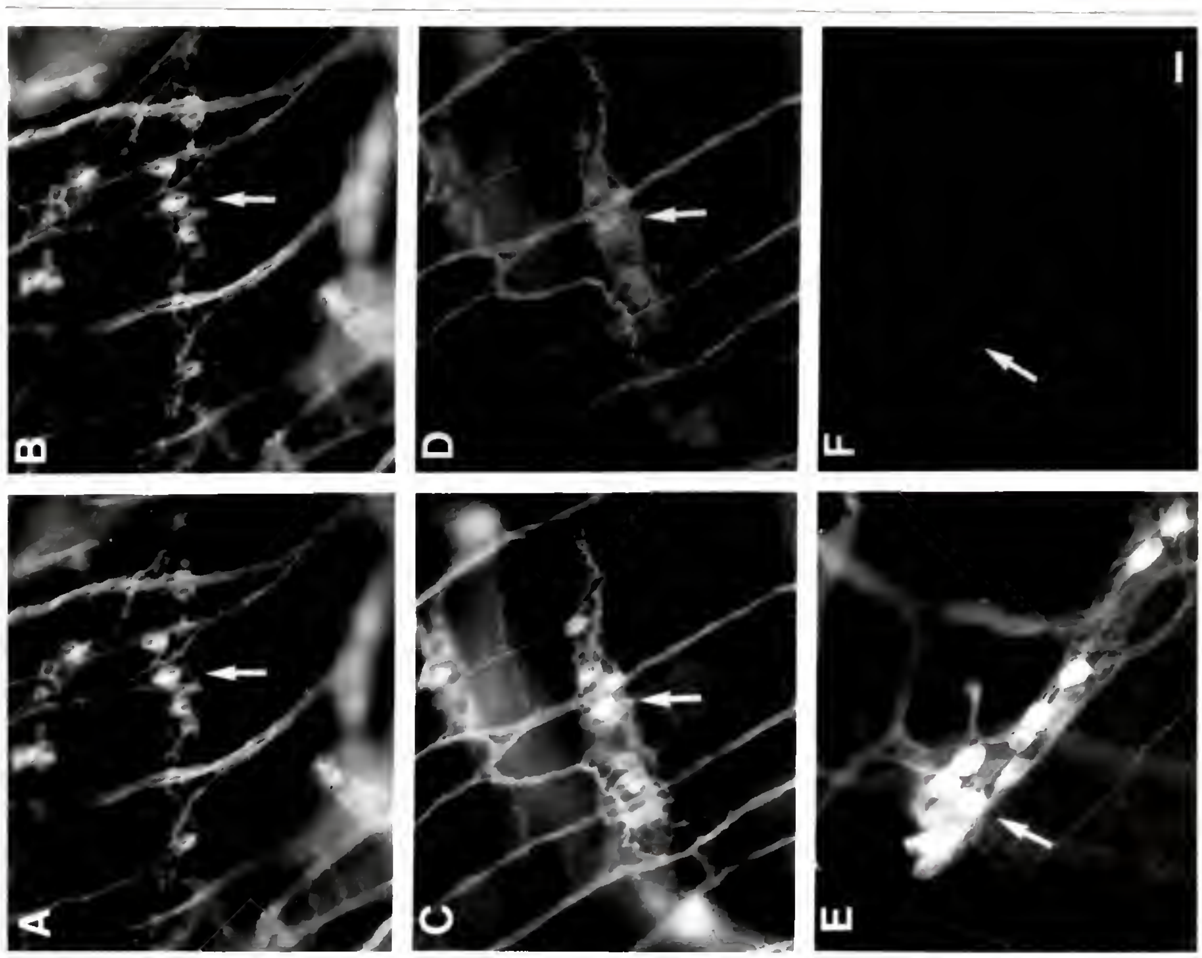

Figure 4-21. Double-label immunofluorescence of myenteric neurons stained with Anti-NF-M KE and RMO59. Whole mounts of myenteric plexi stained with anti-NF-M KE (A, C, and E) and RMO59 (B, D, and F). Anti-NF-M KE stains myenteric neurons in all ages. Neonate intestine (A and B) shows comparable positive immunostaining in neurons (arrows) with anti-NF-M KE and RMO1. Intestine from 7 day old rats (C and D) and adult (E and F) demonstrates a decreased, but detectable RMO59 signal in myenteric neurons (arrows). Scale bar = 20 μm.

whose clustered endings often appeared to terminate near the NF-H immunopositive small Golgi cells (Figure 4-8). With the exception of an occasional Lugaro cell (Figure 4-9), prominent neuronal cell types other than Purkinjes and small Golgis (i.e., granule, basket, stellate, and large Golgi cells) were not seen with neurofilament antibodies in rat cerebellum.

Cat cerebellum was examined to see if these findings were present across species. Nearly all cerebellar neuron types (Purkinje cells, basket cells, stellate cells, Lugaro cells, large Golgi neurons and even granule cells) were clearly NF-H positive in the cat, except that small Golgi cells were not seen using the anti-NF-H KSP serum (Figures 4-10 and 4-11). It may be of note that although Purkinje cells and their axons were even more prominently visible in the cat, the basket cell axon pinceaus were routinely not seen using the anti-NF-H KSP serum (Figure 4-10), though they could be seen with other NF triplet antibodies.

Hippocampus. Another prominent example of a subunit distribution difference was found in the hippocampus. The dentate granule cells and their axonal projection to the Ammon's horn pyramidals, the mossy fibers, were consistently more reactive with antibodies against NF-M than for NF-H or NF-L (Figure 4-12). Although NF-M was apparently more abundant in these cells and their fibers, it was also present in some other hippocampal interneuron cell bodies, though not as much as NF-L and NF-H. The pyramidals of Ammon's horn were

most reactive to NF-H and NF-L antibodies, especially the anti-NF-H KSP serum (Figure 4-13).

Cerebral cortex. The pyramidal cells of the cerebral cortex were also labelled by NF triplet antibodies. Subtle differences in staining occurred between cortical regions and layers, but these differences appeared to correlate with the numbers of pyramidal type neurons. In general the larger pyramidal cells of layer V were most completely labelled (Figure 4-13).

Olfactory neurons. NF triplet antibodies were quite good markers for several type of neurons in the olfactory pathways, including primary sensory neurons in the olfactory epithelium as well as mitral and tufted cells in the olfactory bulb (Figure 4-13). Although labelling occurred for NF-M, NF-L and NF-H usually reacted more strongly with cell bodies of primary and secondary neurons as well as the large dendritic processes in the glomeruli of the olfactory bulb. We saw labelling of primary olfactory neuron dendritic knobs, cell bodies and axons with antibodies against all three NF triplet proteins (Figure 4-13).

Retina. The retinal ganglion and horizontal cell types of both the rat and cat were stained by the NF fusion protein antibodies. Tissue was prepared as a whole mount and various cell types and fibers were examined by focusing through the layers. Not only did they appear larger, but the morphology

of the horizontal cells and their processes were more clearly defined in the cat than the rat (Figure 4-14).

Inner ear. Another area of the rat nervous system in which our NF antibodies detected unexpected findings was in the organs of the inner ear, including the spiral ganglion of the cochlea and the vestibular apparati (Figures 4-15 to 4-18). Afferents to vestibular organs (ampullae, saccule and utricle) were strongly labelled and often appeared to form a calyx at the base of the hair cells; in some cases the hair cells themselves seemed to be stained (Figure 4-16). The vestibular ganglion neurons appeared to be a heterogeneous population based on the spectrum of NF immunoreactivity (Figure 4-16). A similar finding was more clearly documented in the spiral ganglion (Figures 4-17 and 4-18). Approximately 90% of the neurons (type I) of the spiral ganglion send myelinated afferents to the inner hair cells of the Organ of Corti, whereas the remainder (type II) send unmyelinated processes to the outer hair cells. Antibodies directed against the KEP domain of NF-H stained only the type II spiral ganglion neurons, which are also noticeably smaller than type I neurons; all other NF triplet antibodies, however, reacted with both type I and II neurons (Figures 4-17 and 4-18).

Myenteric neurons. As a result of using antibodies which we had epitope-mapped using the fusion proteins, we were also able to extend some previous findings in the enteric nervous system (Table 4-2). Most myenteric plexus neurons from adult

TABLE 4-2. DEVELOPMENTAL PROFILE OF NEUROFILAMENT IMMUNOREACTIVITY IN MYENTERIC NEURONS

	Brain		Myenteric Plexus				
Antibodies	0 day	Adult	0 day	4 day	7 day	21 day	Adult
Polyclonals							
Anti-NF-M KE	+	+	+	+	+	+	+
Anti NF-H KSP	+	+	+	+	+	+	+
Anti NF-H KEP	+	+	+	+	+	+	+
Anti NF-L (H297)	+	+	+	+	+	+	+
Monoclonals							
NN18	+	+	+	↓	-	-	-
RMO1	+	+	+	↓	-	-	-
RMO59	+	+	+	+	↓	↓	↓
5C6	+	+	+	+	+	+	+
1G12	+	+	+	+	+	+	+
1G9	+	+	+	+	+	+	+
5G9	+	+	+	+	+	+	+
3H11	+	+	+	+	+	+	+

(+) = positive immunoreactivity, (-) = negative immunoreactivity, (↓) = decreased immunoreactivity as compared to brain controls.

rats demonstrated neurofilament staining with antibodies directed against different subunits and domains. However, as previously shown (Eaker et al., 1991) enteric neurons from adult rats stain weakly with the NF-M monoclonal NN18 compared to other NF-M, NF-L or NF-H antibodies (Figure 4-19). RMO1 and RMO59, now shown to recognize the same segment of NF-M as NN18, also produce less staining of adult intestine (Figures 4-20 and 4-21, respectively). Neonatal myenteric neurons were examined with the same neurofilament antibodies, but were stained by all of them, including NN18 (Figure 4-19), RMO1 (Figure 4-20) and RMO59 (Figure 4-21). Myenteric neurons were observed to be more tightly packed and smaller in neonates as compared to adult. Intestinal tissues from four-day-old and, to a greater extent, seven-day-old animals displayed

noticeably decreased, or even absent, neuronal immunoreactivity with NN18, RMO1 and RMO59 (Figures 4-19, 4-20 and 4-21, respectively), while maintaining positive staining with the other antibodies. By day 21 the morphology and staining of myenteric neurons was identical to that seen in adult (Table 4-2). This epitope-masking event was seen in both Zamboni-fixed whole mounts and unfixed cryostat sections of intestine, but not in brain controls.

Discussion

We have found a widespread distribution of neurofilament positive cells and fiber tracts in nearly every major area of the adult rat nervous system (Table 4-1). We have demonstrated staining differences based not only on phosphorylation of the KSP domains, as has been previously described throughout the literature (Sternberger and Sternberger, 1983; Shaw et al., 1986; Lee et al., 1986b), but also on a differential distribution of the NF-L, NF-M and NF-H subunit proteins and some of their domains. Apparent differences between the NF subunit and domain distributions are likely to reflect the actual difference in protein concentrations in axons versus cell bodies. Concentration differences would explain why antibodies with non-repetitive epitopes (NF-L, NF-M KE and NF-H KEP) would appear to prefer axons. The resistance of these staining distributions to alkaline phosphatase treatments supports this conclusion.

Several other new patterns of neurofilament expression were discovered in the rat cerebellum, hippocampus, spiral ganglion and the myenteric plexus.

A prominent cell type in the granular layer of the vestibulocerebellum of rat was conspicuously immunoreactive against all NF-H antibodies, but had significantly less reactivity against NF-L antibodies and a nearly undetectable signal for NF-M. The cells were immunonegative for GABA. They often appeared to co-distribute with nodular type mossy fiber terminals as originally described by Brodal and Drablos (1963). Although there are some descriptive discrepancies, we believe that these cells are those identified by Palay and Chan-Palay (1974) as small Golgi neurons, and as "pale" cells by Altman and Bayer (1977) and Sturrock (1990). Although large Golgi cells have been described as GABAergic (Gabbott et al., 1986), these cells were immunonegative for GABA. The small Golgi cell presents an interesting phenomenon in which there is clearly intense staining for NF-H, but nearly none for NF-M. When cat cerebellum was examined with our antibody panel, most cell types were easily detected; the small Golgi neuron, however, if present, was not immunoreactive for neurofilament proteins. Differences in the intermediate filament complement of certain cell types across species have been reported (Shaw and Weber, 1984; Lee et al., 1986a). We have also found staining of a subpopulation of parallel fibers in the rat cerebellum, a finding either unseen or seen but

often ignored or misrepresented in data presented by many previous investigators (Matus et al., 1979; Trojanowski et al., 1985; Bignami and Clark, 1985; Marc et al., 1986; Langley et al., 1988; Dautigny et al., 1988; Vitadello and Denis-Donini, 1990; Roussel et al., 1991; Kaplan et al., 1991). Furthermore, we were able to visualize some granule cells staining with anti-NF-H KSP serum in cat cerebellum. Previous studies have indicated a transient developmental expression of NF-L and NF-M in granule cells, but only α-internexin has been found in the adult (Cambray-Deakin and Burgoyne, 1986; Chiu et al., 1989). Our ability to detect NF-H in the granule cell bodies in mature animals, adds further evidence that at least a subpopulation of parallel fibers are likely to contain NF-H. All staining patterns in the rat were verified on two different strains (Sprague-Dawley and Long-Evans) as well as on both fixed and unfixed tissue; none of our findings were strain-dependent nor altered by fixation.

Examination of the hippocampus with numerous members of our NF antibody panel, allowed detection of a differential subunit distribution in the rat hippocampus. Specifically, NF-M was found to be more prominent in the dentate granule cells and mossy fibers. In a nearly complementary pattern, NF-H and NF-L were more obviously present in the cell types other than dentate granule cells, particularly in the pyramidal neurons of Ammon's horn and in the various interneurons of the hippocampus.

Other major areas of the nervous system including cerebral cortex, olfactory epithelium, olfactory bulb and retina were well stained by our NF fusion protein antibodies. Cortical pyramidal cells, especially those of layer V, were most conspicuously labelled in the cerebrum. Primary sensory neurons of the olfactory epithelium were labelled, but not uniformly. The majority of cells did not stain, but those that did label often were strongly reactive not only in the soma, but in the processes as well. We saw labelling of primary olfactory neuron dendritic knobs with NF-H antibodies as reported in the literature (Bruch and Carr, 1991), but we also saw staining of cell bodies and axons with antibodies against all three NF triplet proteins (Figure 4-13). Previous studies have described the developmental and cell-type specific complement of intermediate filaments in the retina (Shaw and Weber, 1983 and 1984). Similar to what had been described, the rat retinal ganglion and horizontal cell types were stained by the NF fusion protein antibodies. These data were extended by examining cat retinal whole mounts, which had an identical staining pattern; it may be of note that compared to the rat the morphology of horizontal cells and their processes are more clearly defined in the cat.

In the spiral ganglion we found that markers against NF-H KEP domain selectively identified the type II neurons only. Other NF antibodies recognize all of the spiral ganglion neurons (type I and II). Similar findings have been reported

for phosphate-dependent NF-H KSP epitopes (Lawson et al., 1984; Berglund and Ryugo, 1991). Our findings are substantially more intriguing because they are not likely to involve a phosphorylation event due to the nature of the KEP domain. Without a phosphorylation event, epitope-masking might involve an as yet undescribed post-translational modification of NF-H, or perhaps represents masking by binding of an associated protein. The simplest explanation, however, may be that the KEP pattern most accurately describes the NF-H distribution, whereas the KSP pattern represents a cross-reactivity with NF-M. Further studies will need to be done to determine the actual NF-H distribution. We also describe NF staining not only of afferent endings on hair cells of the vestibular organs, as has been previously reported (Kuijpers et al., 1991), but also in vestibular organ hair cells themselves. Although NF-M expression has been reported to occur transiently during cochlear hair cell development (Hasko et al., 1990), it has not been reported in adult hair cells.

Using our array of domain-specific polyclonal and monoclonal NF tail antibodies we have been able to survey the rat and cat nervous systems' neurofilament subunit distribution. These probes have demonstrated differences in neurofilament staining patterns not due to phosphorylation. A general difference in the pattern of somatodendritic versus axonal staining has been systematically clarified by using antibodies against NF-M and NF-H regions outside the KSP

phosphorylation repeats. These markers have now not only revealed the previously poorly-described small Golgi cell of the rat cerebellum, but have also indicated its unusual pattern of neurofilament subunit expression. Our antibodies have also distinguished a subpopulation of parallel fibers of the cerebellum that contain NF-L, NF-M, NF-H and α-internexin. Our carboxyterminal subunit-specific probes have also allowed detection of an apparent distribution difference for NF-M from NF-H and NF-L in the rat hippocampus. Furthermore, we have revealed a novel differential distribution of the NF-H KEP domain in type II, but not type I spiral ganglion neurons. Considering that the KSP domain is shared by NF-M and NF-H, but that KEP domain is unique to NF-H, the least complicated explanation of these results is that the KSP staining represents a cross-reactivity artifact. This difference is not likely to be due to a phosphorylation event. Therefore, if it is not a cross-reactivity artifact, then it may represent another post-translational process or epitope-masking by binding of an associated protein. Examination of the NF-H KEP domain was not specifically possible until this project developed the anti-NF-H KEP serum and the 3G3 and 5B8 monoclonals. The monoclonal antibodies NN18, RMO1 and RMO59, which were all epitope-mapped to the same segment of NF-M using our fusion proteins, have confirmed an epitope-masking event that seems to occur between birth and postnatal day seven in rat myenteric neurons. Several other groups have

described atypical staining patterns with neurofilament antibodies whose epitopes were unknown (Goldstein et al., 1983; Vitadello et al., 1986, 1987; Thorey and Seifert, 1989). Our panel of epitope-mapped antibodies have made previous data more conclusive, revealed some intriguing new phenomena and will likely be useful in future immunohistochemical studies.

CHAPTER 5
IMMUNOHISTOCHEMICAL ANALYSIS OF NF DISTRIBUTION IN PATHOLOGICAL HUMAN BRAIN

Introduction

In many neurodegenerative disorders diseased or dying neurons contain diagnostically significant inclusion bodies. Several dementias including Alzheimer's disease (AD) and progressive supranuclear palsy (PSP) (Steele et al., 1964), as well as Pick's disease, are notorious for their intracellular neurofibrillary tangles (NFTs). Another prevalent neurodegenerative disorder, Parkinson's disease is characterized pathologically by a loss of dopaminergic substantia nigra neurons and by the presence of intracytoplasmic inclusions known as Lewy bodies. Lewy bodies are also found in a disorder known as diffuse Lewy body disease (DLD), in which neurons from many brain regions, including cerebral cortex, contain these inclusions. In all cases these pathological inclusions have been associated with cytoskeletal proteins. Initial work using antibodies on the tangles of AD implicated neurofilaments as a major component of the paired helical filaments (PHFs) which comprise the tangle. The likelihood of neurofilament proteins being an actual component of PHFs has been increasingly downplayed over

the last several years as evidence mounted that the initial correlation was due to a cross-reactive epitope shared by NFs and tau protein, which has been biochemically isolated from PHFs (Goedert et al., 1988; Schmidt et al., 1990). In addition ubiquitin can be examined as a distinct antigenic determinant most concentrated in the NFT lesions along with aberrantly phosphorylated isoforms of tau (Shaw and Chau, 1988; Lee et al., 1991). Although the NFTs of PSP appear to differ from those of AD, NFs are unlikely to be the primary component of that lesion (Schmidt et al., 1988). Lewy bodies, however, do appear to be predominantly composed of neurofilament proteins (Hill et al., 1991). This study attempted to use our well-characterized, epitope-mapped panel of antibodies against neurofilament proteins to further characterize the presence or absence of multiple neurofilament domain epitopes in the tangles of AD and PSP, as well as in Lewy bodies.

Methods

Paraffin blocks of fixed human tissue were obtained from Drs. William Ballinger (Shands/Gainesville VA Neuropathologist) and John Q. Trojanowski (Neuropathologist at University of Pennsylvania). As many cases as possible were selected with various post-mortem intervals and fixation methods (Table 5-1). Paraffin sections (20 μm) were cut and mounted onto slides, dried and stored at room temperature.

TABLE 5-1. PARAFFIN BRAIN BLOCKS USED FOR NEUROPATHOLOGY

KEY:
Diagnosis: (N)ormal, (A)lzheimer's, (P)arkinson's, (PSP) progressive supranuclear palsy, (AA) Amyloid Angiopathy, (DLD) diffuse Lewy body disease;
Fixative: (F)ormalin, (B)ouin's, (E)thanol;
Region: (F)rontal,(T)emporal or Cingulate Cortex, (H)ippocampus, (B)rainstem;
AINT = Autopsy interval post mortem (hours); Age (years);
Race: (C)aucasian or (N)egro; Sex: (M)ale or (F)emale

Case #	Diagnosis	Fixative	Region	AINT	Age	Race	Sex
Cases from Dr. Ballinger - Shands/Gainesville VA:							
A89-9	A	F	H	18	55	C	F
A25-88	A	F	F	18	77	C	F
A88-004	N	F	T,H	21	68	N	F
A88-008	N	F	H	7	53	C	F
A88-50	A	F	F	16	74	C	M
A88-68	N	F	H	6	67	C	M
A13-87	N	F	H	7	63	N	M
A22-87	N	F	H	26	74	C	M
A89-87	A,P,AA	F	F,H,B	14	90	C	M
A23-87	A	F	F	18	66	C	M
A31-87	N	F	F	26	66	N	M
A34-87	N	F	H	16	55	C	M
A38-87	N	F	H	15	52	C	M
A40-87	N	F	H	4	67	C	M
A25-86	A,AA	F	F,H	4	75	C	M
A86-133	A	F	H	27	68	C	M
A25-85	A	F	T,H	13	75	C	M
A72-84	A	F	H	28	66	C	M
A83-84	A	F	H	10	84	C	M
A91-84	A	F	H	1	61	C	M
Cases from Dr. JQ Trojanowski - Univ. of Pennsylvania:							
86-88	N	B	H	8	66	-	-
86-215	A	B	H	12	83	-	-
91-8	A	E	T	10	71	-	-
91-63	A,P	E	H	3.5	74	-	-
91-36	P	E	B,C	20	63	-	-
91-22	P,DLD	E	C,T	14.5	83	-	-
86-206	PSP	B	B	6	75	-	-
89-244	PSP	E	B	5	78	-	-

Standard immunohistochemistry using a biotin-avidin peroxidase procedure (Vectastain Elite - Vector Laboratories) following deparaffinization. After staining and development were complete sections were dehydrated, sometimes counterstained with Harris Hematoxylin, mounted with Permount and stored at room temperature.

The entire panel of polyclonal and monoclonal fusion protein antibodies described earlier was used to screen the cases. In addition, control antibodies included: our ubiquitin monoclonal (UBI-1) as well as a tau monoclonal (T14) and a well described monoclonal (RMO32) for detection of Lewy bodies from Dr. J.Q. Trojanowski.

Results

Of all cases stained irregardless of the diagnosis the fixative seemed to have the most effect on success with antibody staining. The ethanol-fixed material reacted best. Bouin's fixation was also often good for antibody staining. The success with formalin fixation, however, was highly variable. Because we had relatively few cases fixed by the preferred methods, only a very limited number of slides were actually available for proper evaluation of inclusion staining.

Although most of the fusion protein antibodies, were at least weakly reactive against normal neurofilaments in human tissue, the anti-NF-H KSP and anti-NF-H KEP rabbit

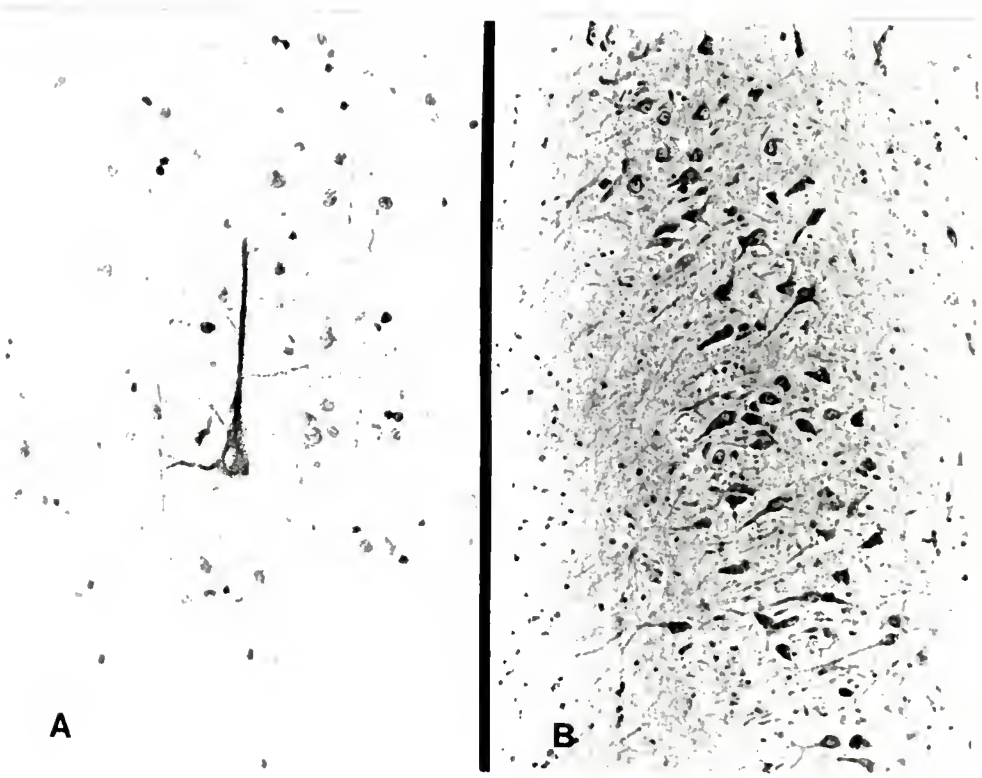

Figure 5-1. Immunohistochemical staining of normal human brain. A: Normal human cerebral cortex layer V pyramidal neuron stained by NF-M KE monoclonal 3H11. B: Normal human hippocampus CA1 pyramidal neurons stained with anti-NF-H KEP polyclonal. Approximate magnification of A = 200x; B = 100x.

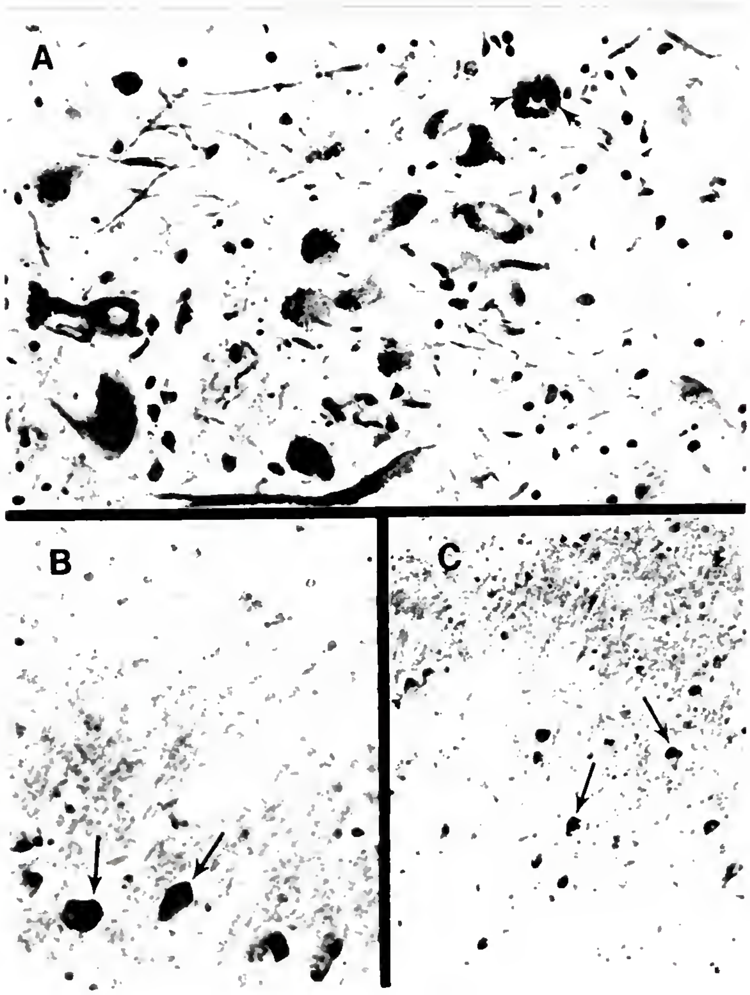

Figure 5-2. Immunohistochemical staining Lewy bodies. A: Anti-NF-H KEP positive Lewy bodies (arrowheads) dopaminergic substantia nigra neuron of a Parkinson's patient. B and C: RMO32 positive Lewy bodies (arrows) in cingulate cortex from a DLD patient. Approximate magnification of A = 200x; B = 200x; C = 63x.

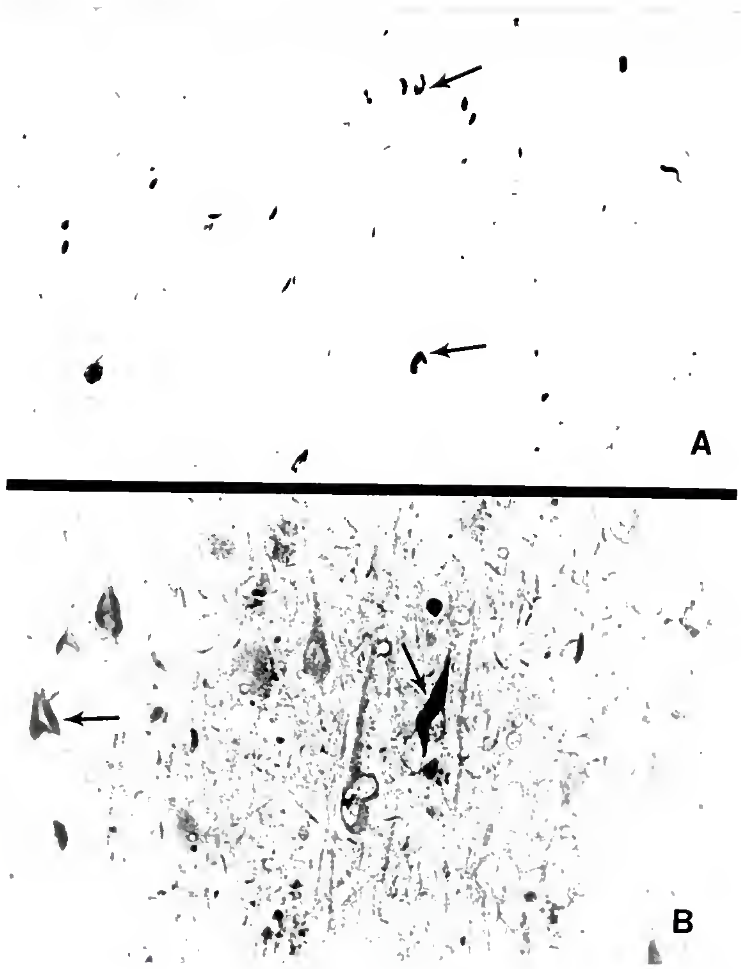

Figure 5-3. Immunohistochemical staining of cerebral cortex neurofibrillary tangles (NFTs) of Alzheimer's disease by tau and ubiquitin monoclonal antibodies. A: Tau monoclonal T14 positive profiles of NFTs (arrows). B: Ubiquitin monoclonal Ubi-1 positive staining of NFTs (arrows). Approximate magnification of A = 63x; B = 200x.

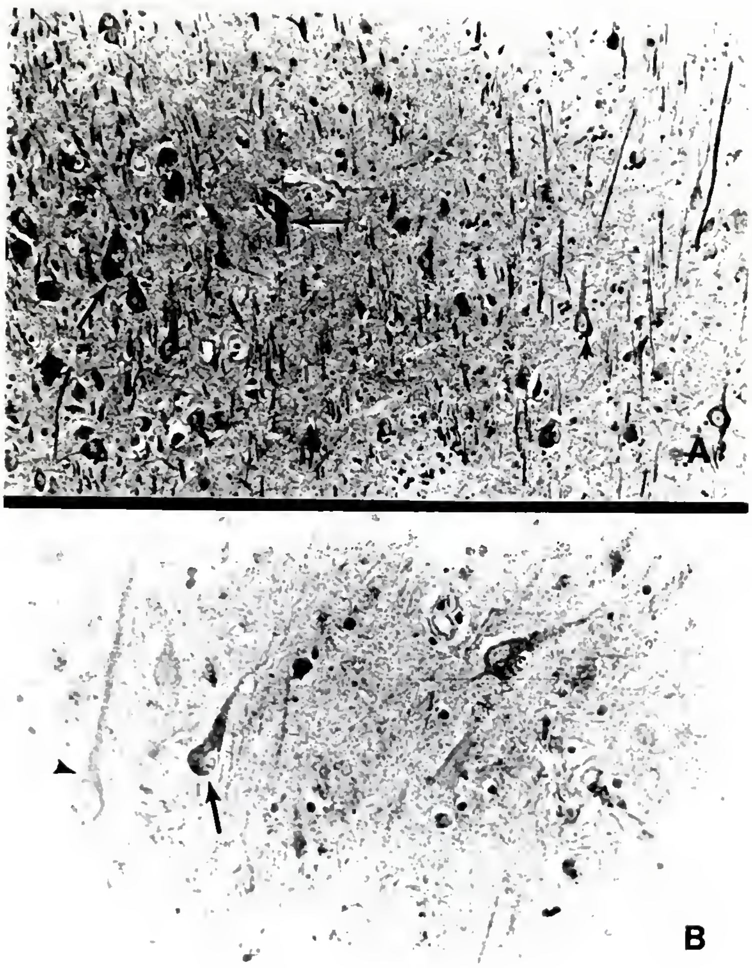

Figure 5-4. Immunohistochemical staining of cerebral cortex neurofibrillary tangles (NFTs) of Alzheimer's disease with neurofilament fusion protein antibodies. A: Anti-NF-H KSP polyclonal staining of both normal pyramidal neurons (arrowhead) and NFTs (arrows). B: Anti-NF-M KE polyclonal staining of a normal pyramidal neuron (arrowhead) and a NFT (arrow). Approximate magnification of A = 63x; B = 200x.

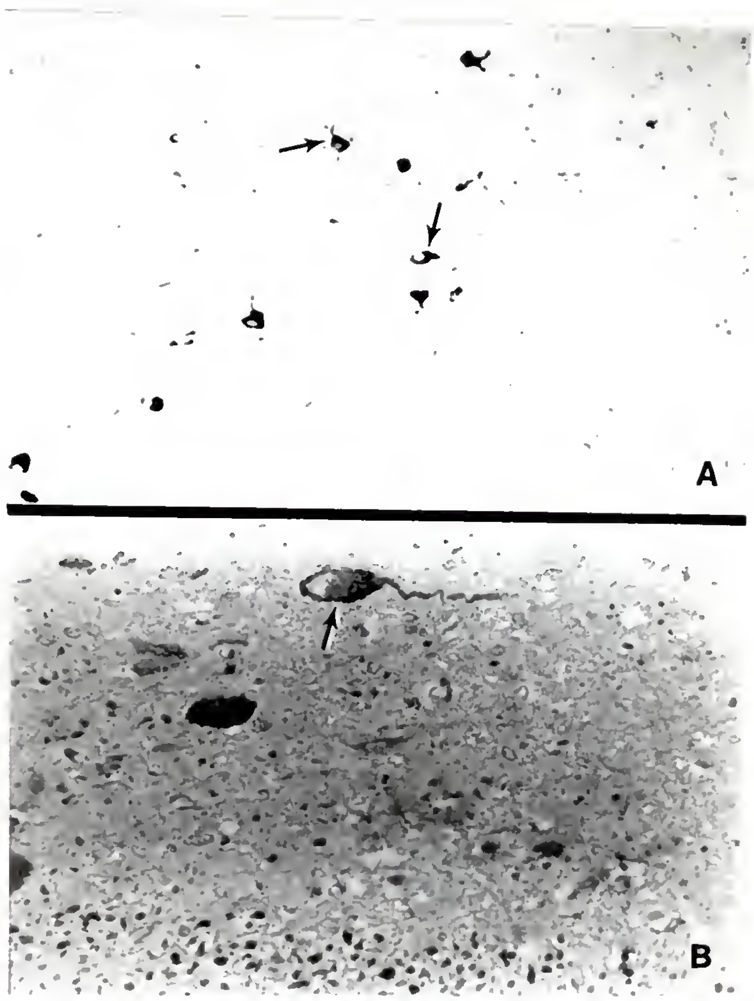

Figure 5-5. Immunohistochemical staining of brainstem NFTs of progressive supranuclear palsy (PSP). A: Strongly positive staining of PSP NFTs with tau monoclonal T14 (arrows). B: Weakly positive anti-NF-H KSP polyclonal staining of PSP substantia nigra neurons (arrow). Approximate magnification of A = 63x; B = 200x.

polyclonals, the NF-H KSP monoclonal N52 and a few of the monoclonals against the NF-M KE segment (5C6, 1G12, 3H11 and 4H4) worked best (Figure 5-1). These same antibodies, especially 3H11, were most useful for detecting Lewy bodies of Parkinson's disease patients (Figure 5-2A). A monoclonal already described as a marker for Lewy bodies (RMO32) was obtained and used as a control. RMO32 appears to detect an abnormal neurofilament epitope (perhaps an aberrant phosphorylation site) and does not react very strongly with normal neurofilaments. This antibody is also highly reactive against the Lewy bodies found in DLD (Figure 5-2B and C). Tangles from AD (Figures 5-3 and 5-4) and PSP (Figures 5-5) were identifiable from morphology, but the specificity of detection of degenerating cells with the neurofilament antibodies (Figures 5-4 and 5-5B) was usually not significantly different from that seen for normal unaffected neighboring neurons. In contrast we did use a ubiquitin (UBI-1) monoclonal and a tau (T14) monoclonal as control incubations to verify the presence of pathology in adjacent sections (Figures 5-3 and 5-5A).

Discussion

After testing a large panel of epitope-mapped neurofilament monoclonal and polyclonal antibodies on both normal and pathological human tissue, we saw that most non-cross-reactive neurofilament antibodies stain degenerating

neurons as well as apparently healthy ones. With the exception of the Lewy bodies of Parkinson's disease, antibodies which stain pathological inclusions more strongly than the background of neurofilaments in normal neurons and fibers are likely to either recognize altered neurofilament epitopes (i.e., sites that are aberrantly phosphorylated) (Schmidt et al., 1988) or cross-react with MAPs, actin or actin associated proteins (Schmidt et al., 1989). Lewy bodies, however, do appear to contain neurofilament subunits (Hill et al., 1991).

Our NF-M KE monoclonals 5C6, 1G12, 3H11 and 4H4 as well as the NF-H KSP and KEP polyclonals were most reactive with normal somatic and axonal human neurofilaments as well as with Lewy bodies. In addition the NF-H monoclonal N52 was also quite reactive on human NFs and on Lewy bodies. By characterizing which of our epitope-mapped antibodies are reactive with pathological inclusions, we have confirmed and extended the published reports that much more of neurofilament tails are likely to actually be a major component of Lewy bodies (Hill et al, 1991). Our results further support the increasing evidence that neurofilaments, though present, are not principal elements of the neurofibrillary tangles found in diseased neurons of progressive supranuclear palsy and Alzheimer's disease.

CHAPTER 6
SUMMARY AND CONCLUSIONS

NF Fusion Proteins

The strength of this entire study ultimately relates back to the initial construction of cDNA clones coding for putative NF-M and NF-H carboxyterminal tail domains which were previously described based upon sequence analysis (Shaw, 1989). Using these constructs we were able to express fusion proteins containing distinct segments of the NF tails and to even further dissect these regions using proteolytic methods to generate fusion protein fragments. Together the fusion proteins and fragments have served as substrates for epitope mapping existing antibodies, including two widely used monoclonal neurofilament antibodies, NN18 and N52. These fusion proteins have also made excellent antigens to generate new polyclonal and monoclonal antibodies of predefined specificities. In addition, some of the methods we have employed here should be generally useful for further studies of neurofilament proteins. For instance the hydroxylamine NG cleavage can be used to delete parts of the extreme C-terminus of NF-M and to isolate the extreme C-terminal fragments from neurofilaments in vivo, and should also work on a wide variety of species since the two NG cleavage sites are conserved in

all known NF-M molecules, even in species as evolutionarily distant as Torpedo (Linial and Scheller, 1990).

NF Fusion Protein Antibodies

The fusion protein antibodies represent the second major strength of this study. Not only have we been able to map the epitopes of existing neurofilament antibodies using the forementioned fusion proteins, but we have also generated a new panel of subunit and tail domain specific polyclonal and monoclonal antibodies by using the fusion proteins as antigens. The fusion protein antibodies are a first step in the direction of not only characterizing NF distributions in tissue sections, but will also be of considerable use both for biochemical studies of neurofilaments and possible associated proteins as well as for cell biological investigations.

Immunohistochemical Distribution of NF-M and NF-H

With the panel of biochemically characterized fusion protein antibodies in hand, the most obvious next study was to examine tissue sections. Because the fusion proteins contained rat sequences, we looked first at rat nervous system tissues. The antibodies proved to be excellent for immunohistochemistry and we were able to do a survey of major nervous system structures including cerebral cortex, olfactory bulb, hippocampus, retina, midbrain, brainstem, cochlear and vestibular organs, dorsal root ganglia, spinal cord and

myenteric plexus. Of particular interest were our findings in the cerebellum. We found that a subpopulation of parallel fibers were in fact immunopositive for α-internexin, NF-L, NF-M and NF-H (a finding unseen by most previous studies) and that the vestibulocerebellar lobules were enriched in a previously undescribed NF-H immunopositive cell in the granular layer. After considerable efforts to identify these cells, we concluded that they were likely to be the small Golgi neurons described Palay and Chan-Palay in 1974. These neurons were of further interest because of their unusual staining pattern which suggested that they have much more NF-H than NF-M or NF-L. Though subunit differences such as this are not a well-described nor common phenomenon, we also examined a complementary distribution of NF-M from NF-H and NF-L in the hippocampus. Upon looking at another species, cat, we found that even though our antibodies worked well for discerning many cerebellar cell type in cat tissues, these small Golgi cells were not a detectable cell type immunohistochemically using the same antibodies. In addition our new NF-H KEP antibodies have indicated that in type I spiral ganglion neurons of the rat NF-H may be modified in a previously undescribed manner; due to the characteristics of the KEP domain this modification is unlikely to be a phosphorylation event. Finally we were able to extend previous epitope-masking results in myenteric neurons with the NF-M KE monoclonal NN18. By epitope mapping two other monoclonals to

the same domain as NN18 by immunoblotting, we successfully predicted a similar epitope masking effect for these two antibodies in the myenteric plexi; we were also able to show that this masking event occurs between postnatal days 4 and 7.

NF Distribution in Pathological Human Brain

In this immunohistochemical investigation of normal pathological human brain tissues we used our epitope-mapped antibodies to confirm and extend published reports looking at pathological inclusions in Alzheimer's disease, progressive supranuclear palsy and Parkinson's disease. Although we found that neurofilament antibodies could stain all pyramidal neurons (healthy and degenerating), we could distinguish tangles based on morphology. Our studies agree with the vast current literature indicating that neurofilaments are unlikely to be a specific component of the neurofibrillary tangles found in Alzheimer's disease. Similarly we found no extraordinary reactivity with tangle from degenerating neurons of progressive supranuclear palsy cases. We did, however, extend previous findings by showing that additional tail domains from NF-M and NF-H are present in the Lewy bodies of Parkinson's disease.

Conclusions

As a starting point for examining neurofilament biology, we took a systematic approach by generating cDNA constructs

and producing purified fusion proteins to probe existing markers. This project has generated new and powerful probes that enabled us to describe more accurately than previously possible the relative distributions of NF-M and NF-H in various neuronal cell types. Our probes revealed unique neurofilament subunit distribution pattern in the cerebellar Golgi cells as well as hippocampal dentate granule cells and mossy fibers. We were able to demonstrate both a subpopulation of NF immunoreactive parallel fibers in rat and granule cells in the cat cerebellum. Furthermore our new antibodies against the previously indistinguishable NF-H KEP segment have indicated that this region may be a functionally variable site in vivo; the NF-H KEP domain is selectively missing from type I spiral ganglion cells. Similarly, we were able to extend previous observations concerning the site and time course of an epitope masking event on NF-M in myenteric neurons of the rat. In addition, we were able to confirm earlier reports concerning the presence or absence of neurofilaments in various pathological inclusions. Together these findings are indicative of the significantly increased power of interpretation these new probes will yield. Clearly the information and tools produced by the study will be useful for future biochemical and cell biological investigations of neurofilament function.

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BIOGRAPHICAL SKETCH

Jeffrey Mark Harris was born in Jacksonville, Florida, on January 10, 1965. His first exposure to neuroscience took place during the summer following his junior year of high school when he participated in the 1982 Student Science Training Program at the University of Florida; this science research program placed him in the Department of Neuroscience in Dr. Adrian Dunn's laboratory, where he worked on the central serotonergic response to stress. He continued this research project and presented it to win second place at the 1983 Florida State Science Fair. As a University of Florida undergraduate he worked in Dr. Steve Childers' laboratory on the characterization of brain opioid peptide receptors, which was the thesis topic for his Bachelor of Science in Neuropharmacology. He received his B.S. with high honors in December 1986 and continued research in the Department of Neuroscience as a postbaccalaureate student in the laboratory of Dr. Gerry Shaw. In August 1987 Jeff entered the M.D./Ph.D. Program at the University of Florida and has performed the work contained in this dissertation in Dr. Shaw's laboratory. Having finished his Ph.D. program, Jeff will complete the last two years of medical school at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gerry Shaw

Gerry Shaw, Chairman
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Robert Nicholls

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Scientist of Neuroscience

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